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NON-SYMBIOTIC NITROGEN FIXATION IN SOIL *)

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INTRODUCTION

The evaluation of the significance of non-symbiotic nitrogen fixation in soil has been impeded by two factors:

1. Nitrogen gains are usually small. In soils of average nitrogen content, increases of 20–60 pounds of nitrogen per acre per year can only be verified by statistical treatment of a large number of Kjeldahl analyses.

2. Under field conditions, fluctuations in nitrogen content of the soil occur as a result of leaching and drainage, increases from atmospheric sources of fixed nitrogen, denitrification, or nitrogen fixation. The first two may be determined or eliminated by special procedures, but denitrification must be estimated. The net result of fixation and denitrification may then be determined, but a quantitative estimate of each individual process will be obscured.

In a previous communication [16]), studies on denitrification were reported which involved the use of a tracer technique. It was found that, in poorly aerated soils, denitrification could proceed rapidly and N_2O was one of the major products.

In this report, experiments on non-symbiotic, aerobic nitrogen fixation are described. By using an atmosphere labeled with N_2^{15} it was possible to increase greatly the sensitivity by which fixation could be detected when compared with difference methods and also to determine nitrogen fixed directly.

Winogradsky was the first to demonstrate nitrogen fixation by a free-living micro-organism, *Clostridium pasteurianum*. Soon thereafter, Beijerinck isolated an aerobic nitrogen-fixing

*) This investigation was supported in part by a contract with the United States Atomic Energy Commission.

organism, *Azotobacter*. The introduction of tracer techniques by Burris and Miller[2]) greatly contributed to the study of nitrogen fixation. Kamen and Gest[9]) showed that the photosynthetic bacteria were able to fix nitrogen. Recent developments along these lines have been reviewed by Wilson[17]).

Competitive inhibition of nitrogen fixation by H_2 and N_2O has been investigated by Wyss *et al.*[19]), and Repaske and Wilson[12]). Parker[11]) in a note just published, reported considerably increased nitrogen fixation by *Azotobacter* in cultures aerated with an atmosphere containing 4 per cent oxygen, as compared with cultures aerated with an atmosphere containing 20 per cent oxygen. Wyss *et al.*,[19]) studied the effect of nitrogen tensions on nitrogen fixation by *Azotobacter* under a constant pO_2 (0.2 atmospheres). Their data showed that nitrogen fixation was constant between a pN_2 of 8 atm. and 0.2 atm. Since, in these experiments, the O_2/N_2 ratio varied between 0.4 and 1.0, the suppressing effect of oxygen is apparently independent of the O_2/N_2 ratio between these limits. Probably the suppressing effect observed by Parker depends on the absolute pO_2. Even though the O_2/N_2 ratio used in our experiments was often as high as 0.7, the absolute pO_2 closely corresponded to that found under natural conditions. The data of Wyss *et al.*, suggest therefore that in our experiments nitrogen fixation probably was not suppressed, as compared with fixation in natural soils. More information would be desirable on the effect of the absolute oxygen pressure as well as the O_2/N_2 ratio on nitrogen fixation.

Qualitatively also, the extent of nitrogen fixation by non-symbiotic organisms is not known. Virtanen[15]) stated this clearly in a recent review:

"In verschiedenen Böden ist der jährliche Betrag der durch die freilebenden Bakterien geschaffenen Stickstoffbindung... sehr verschieden. Wir sind über die hierbei im Frage kommenden Mengen noch aüsserst mangelhaft unterrichtet. Schwankungen von ein paar bis 30 Kilogramm pro Hektar und Jahr sind wahrscheinlich."

Although it is possible to make a quantitative evaluation of denitrification and nitrogen fixation by the use of isotopic nitrogen, the cost of the isotope places a limit on the scope of any field experiment which might be designed. The use of a small closed

system such as that resorted to in the present work, represents a deviation from field conditions in order to keep within reasonable costs.

METHODS

In order simultaneously to evaluate nitrogen fixation, denitrification and nitrification in a typical soil under various conditions, the soil was subjected to various treatments and then surrounded with an atmosphere containing N^{15}-labeled nitrogen. Exchange of gases was followed with time, and the soil nitrogen was fractionated and analyzed at the end of intervals varying from 30 to 48 days.

Except for some preliminary experiments, the bottle and manometer assembly used in earlier experiments on denitrification [16]) was found to be impractical for use in nitrogen fixation studies. Instead, a number of samples were incubated in a large vacuum desiccator containing nitrogen and oxygen and enough KOH to absorb the excess CO_2 produced.

The procedures used in the analysis of the different nitrogen fractions of the soil have been discussed in an earlier communication [16]).

Labeled nitrogen gas was prepared by oxidation of ammonia with alkaline hypobromite. Hypobromite solution was prepared by mixing 10 volumes of a solution containing 10 per cent NaBr and 10 per cent Br_2 with 10 volumes of 40 per cent NaOH, and adding 30 volumes of water. To produce about 22 ml nitrogen gas, 2 m*M* NH_4^+ were admitted to an evacuated bulb and 20 ml hypobromite reagent were then added. The bulb was shaken intermittently until the reaction had gone to completion. The gas produced was displaced with a solution of 20 per cent sodium sulfate in 5 per cent sulfuric acid, the latter also serving to absorb any traces of ammonia that might be left. Although the gas so prepared should have been pure, it was always checked mass spectrometrically. Impurities were never detected. An atmosphere containing about 6 atom per cent N^{15} excess was used. Depending somewhat on the methods used, the minimum enrichment that could be detected was about 0.002 atom per cent excess N^{15}. Since the atmosphere contained about 6 atom per cent excess N^{15}, a dilution of 1 : 3000

could just be detected, or, in other words, if more than 0.03 per cent nitrogen in a fraction was derived from fixation, it could be detected mass-spectrometrically. On the basis of 200 pounds nitrate nitrogen per acre 6 inches deep, the experimental error, due to mass-spectrometric techniques, was about 0.06 pounds N. In the organic fraction, the error was slightly larger. Most of the soil contained about 60 microequivalents organic nitrogen per gram soil. The experimental error was 0.03 per cent, or 0.018 microequivalents nitrogen per gram soil, which is about 0.5 pounds nitrogen per acre 6 inches deep. This error has henceforth been indicated.

All calculations have been made in terms of microequivalents nitrogen per gram *dry* soil. In terms of pounds per acre, one microequivalent nitrogen per gram dry soil equals approximately 28 pounds nitrogen per acre 6 inches deep. Similarly, 1 milligram added organic matter (*e.g.*, alfalfa) per gram of soil is equivalent to about 1 ton per acre 6 inches deep. Since nitrate suppresses nitrogen fixation, initial and final nitrate concentrations have been reported.

Tests for the presence of *Azotobacter* were made using both plate counts and liquid cultures, according to methods described by J e n s e n [5]). He showed that his method is quite reliable although it does not distinguish between cell aggregates and individual cells.

RESULTS

Nitrogen fixation in Yolo soil to which straw was added

Two hundred gram portions of Yolo soil, described more fully in an earlier communication [16]), and 20 g fresh garden soil inoculum were mixed respectively with 200 mg, 400 mg, and 800 mg of straw. As a control, one soil was used without added substrate.

The soils were incubated for 48 days in bottles fitted with manometers, under an atmosphere containing 28 cm N_2^{15} and 20 cm O_2.

From the oxygen consumed, carbon recoveries from the straw were calculated on the basis of a respiratory quotient equal to one, and assuming straw to be 44.4 per cent carbon. These recoveries are shown in Table I. At the end of the experimental period, nitrate, ammonium ion and total nitrogen were determined and the nitrogen of these fractions was assayed for N^{15} content.

TABLE I

Substrate utilization
Straw added to Yolo soil. Recoveries calculated from oxygen consumed assuming straw to contain 44 per cent carbon and R/Q of 1. Corrected for endogenous respiration (see text)

Straw added milligrams per g soil (tons per acre)	Carbon recovered per cent	
	30 days	48 days
2.0	22	23
4.0	31	38
8.0	32	37

It was apparent, as we had found earlier, that the complete oxidation of straw was very slow. This slow rate was not due to nitrogen deficiency because in all but the soil to which 800 mg straw were added, at least one microequivalent nitrate per gram soil was left at the end of the experiment.

The fixation data are summarized in Table II. The only significant fixation was found in the nitrate fraction. Both total nitrate found at the end of the experiment and "nitrate fixed", *i.e.*, the quantity of fixed nitrogen in the nitrate fraction, calculated from N^{15} excess in that fraction, are shown in the table. The results were somewhat surprising. Although conditions were thought to be favorable for fixation, only a very small quantity of nitrogen was fixed. The appearance of the isotope in nitrate, is interpreted to be the result of the autotrophic oxidation of ammonium ion, the probable initial product of fixation.

In order to eliminate possible suppression of fixation by nitrate, leached Yolo soil was used in subsequent experiments.

TABLE II

Nitrogen fixation with straw as added substrate
200 g Yolo soil including 20 g fresh garden soil inoculum P_W = 20%; pH = 7.6; pO_2 = 15–20 cm; pN_2 = 25–28 cm; NO_3^- = 3.2 microequivalents per g soil. Incubated 48 days at 21°C

Straw added milligrams per g soil (tons per acre)	Nitrate microequivalents per g soil		N_2 fixed lbs. per acre 6″ deep in 48 days
	final (by analysis)	fixed (calculated from isotope content)	
none	3.69	0.025	0.7
1.0	2.71	0.047	1.31
2.0	1.68	0.055	1.54
4.0	0.56	0.066	1.85

Effect of substrate type and level and of nitrate concentration

A number of soil treatments were used to determine under what conditions nitrogen fixation might occur. Nineteen samples were incubated for 46 days in a large desiccator with an atmosphere containing 6.62 atom per cent excess N^{15}. All samples contained 92.5 g Yolo soil, which had been washed twice to remove nitrate, and 15 g fresh garden soil inoculum. (Total dry weight of soil was 100 g.) The initial moisture content was about 19 per cent, but during incubation it fell to about 15 per cent. Both alfalfa and wheat straw were tried as substrates at various concentrations, and different levels of pH and nitrate content were tried.

The results are shown in Table III. Nitrogen fixation, as computed from N^{15} excess in each of the three fractions, ammonia-amide, nitrate, and organic N, is listed separately.

Maximum fixation reached only 0.047 μe N per gram soil (1.32 $\pm$ 0.5 lbs N per acre 6 inches deep). Under most conditions, less than 0.018 μe N per gram soil (0.50 lbs N per acre 6 inches deep) was fixed.

Considering the wide variety of conditions used to induce fixation, these results augmented the doubts created by experiment 1, concerning the prevalence of nitrogen fixation under these conditions. High nitrate concentration was not responsible for the low rate of fixation because little nitrogen was fixed regardless of nitrate concentration. More nitrogen was fixed with alfalfa as substrate than with straw. Since *Azotobacter* will grow on a medium containing only an organic energy source and minerals, the alfalfa presumably provides a more readily available energy source for the nitrogen-fixing organisms than does straw.

Fixation in soils under a "natural" cover

The results of the preceding experiments indicated that little nitrogen fixation occurred in Yolo soil, irrespective of substrate used or nitrate concentration in the soil. Nitrogen fixation in the dried soil used was possibly adversely affected by the slow growth of *Azotobacter* or the absence of a suitable substrate that might be provided by an actively metabolizing population.

Another possibility that had not been tested was the influence of growing plants and their roots on fixation. Although they provide competition for inorganic ions in the soil, plants may support a

TABLE III

Fixation under various levels of substrate, nitrate, and hydrogen ion concentration
100 g Yolo soil including 15 g garden soil inoculum. Incubated 46 days at 21°C

Conditions			Nitrogen fractions						
Substrate milligrams per g soil	Nitrate microequivalents per g soil	pH	Ammonia microequivalents per g soil		Nitrate microequivalents per g soil		Organic N microequivalents per g soil	Total N fixed	
			final	fixed	final	fixed	fixed	microequivalents per g soil	lbs/acre 6″ deep
0	1.1	7.8	0.10	—	2.24	—	0.018	0.018	0.51
2.0 mg straw	1.1	7.8	0.21	0.002	0.55	0.002	—	0.004	0.11
4.0 ,, ,,	1.1	7.8	0.15	0.001	—	—	0.026	0.027	0.76
8.0 ,, ,,	1.1	7.8	0.16	0.004	—	—	—	0.004	0.10
0	1.1	7.8	0.10	—	2.24	—	0.018	0.018	0.51
2.0 mg alfalfa	1.1	7.8	0.44	0.002	2.42	0.006	0.018	0.026	0.71
4.0 ,, ,,	1.1	7.8	0.97	0.005	2.47	0.010	0.030	0.045	1.26
8.0 ,, ,,	1.1	7.8	lost	—	5.48	—	0.021	0.021	0.59
3.0 mg straw	2.3	7.8	0.29	0.002	1.92	0.002	—	0.004	0.10
3.0 ,, ,,	3.4	7.8	0.26	0.002	4.05(?)	0.007	—	0.009	0.25
3.0 ,, ,,	5.6	7.8	0.15	0.003	3.73	0.001	0.009	0.012	0.34
3.0 ,, ,,	10.2	7.8	0.65	0.001	8.71	0.001	0.017	0.019	0.53
3.0 mg straw	1.1	4.5–5.1	0.79	0.003	—	—	—	0.003	0.07
3.0 ,, ,,	1.1	5.2–5.6	0.52	0.003	0.05	0.002	—	0.005	0.14
3.0 ,, ,,	1.1	5.8–6.3	0.27	0.001	0.05	0.001	—	0.002	0.04
3.0 ,, ,,	1.1	6.7–7.3	lost	0.006	0.28	0.003	0.038	0.047	1.32
3.0 ,, ,,	1.1	8.4	0.17	—	0.10	—	—	—	—

fixing flora by the decay of dead root material and other plant tissues and possibly by the loss to the soil of soluble substrates from actively photosynthesizing plants.

To test these conditions, a series of soil discs was collected from a permanent lawn on the Davis Campus of the University of California. This lawn, growing on Yolo sandy loam, had not been fertilized for a number of years and was nitrogen deficient. The grass had been cut regularly and most, but not all, cuttings were allowed to decompose *in situ*. Since nitrogen is lost in this porous soil by leaching and is removed in some of the cuttings, either the soil becomes nitrogen depleted, or non-symbiotic fixation must occur. No legumes were found in the sample area. *Azotobacter* cells were found to be present (see Table V). Similar soil discs were collected from a lawn on the Berkeley Campus. Details of the experiment are given in Table IV. Some discs were placed in dishes with the grass up in a normal position, whereas others were inverted to compare a photosynthesizing disc with a "fallowed" or "green manure" disc. The samples were placed in a desiccator to which the following gases were admitted: $N_2^{15} = 23$ cm; $O_2 = 20$ cm; $CO_2 = 3$ cm. The desiccator containing the discs was illuminated during the day with two 20-watt fluorescent lamps, and at intervals with a 150-watt tungsten spotlight which was placed behind a flat flask, approximately 30 mm thick, containing water to absorb infrared radiation.

The "lawns" had a tendency to wilt and die during the first few days, but after part of the leaves had died, and although growth was not vigorous in the desiccator, the grass recovered considerably. (This is also shown by the low nitrate values in these samples as compared with the inverted discs.)

A considerable quantity of carbon dioxide accumulated and was removed by Toepler-pumping the gases over a confining liquid of KOH. Since some of the soils dried somewhat during the experiment, final P_w values have also been recorded.

Results are shown in Table IV. The large quantity of nitrogen fixed in the control soil to which phosphate buffer and sucrose had been added indicated that nitrogen fixation was not inhibited by any intrinsic characteristic of the soil, but rather by the lack of an appropriate substrate. Significant fixation also took place in the inverted discs in which the grass was decaying. It is interesting to note that, as before, almost all nitrogen fixed was detected in the

TABLE IV

Fixation in sod sections

Soil discs, 10 cm diameter, 3–4 cm deep; collected from Davis (Yolo sandy loam, pH = 8.1) and Berkeley campuses (pH = 6.2); P_w (initial) = M.E. Incubated 31 days at 21°C

Treatment	Soil	P_w (final)	Ammonia microequivalents per g soil		Nitrate microequivalents per g soil		Organic N microequivalents per g soil	Total N fixed	
			final	fixed	final	fixed		microequivalents per g soil	lbs. per acre 6″ deep
10 g Yolo soil 7·0 mg sucrose	Davis	3.5	0.66	0.055	0.75	0.028	12.2	12.3	344
Growing lawn, photosynthesizing	Davis	10.4	1.24	—	0.88	0.0004	—	0.0004	0.011
	Berkeley	7.1	1.78	0.004	1.39	0.001		0.005	0.140
Inverted lawn, decaying	Davis	22.7	1.49	0.18	5.44	0.26	—	0.44	12
	Berkeley	20.0	3.79	0.087	7.46	0.047	—	0.134	3.75
Soil with grass removed	Davis	3.6	1.07	0.001	0.99	0.007	—	0.008	0.22
	Berkeley	3.5	0.67	0.001	0.46	0.002	—	0.003	0.085

TABLE V

Character of soils used for comparison of nitrogen-fixing capacity				
Soil	Cover	pH	Nitrate content microequivalents per g soil	*Azotobacter* cells or clusters per g soil
Cowell clay	Sprouting barley	8.0	1.25	30
Rincon clay	Sprouting barley	7.2	0.90	300
Altamont clay loam	Shallow young soil, grass cover	7.6	0.65	—
Manzanita gravelly clay loam	Pasture	5.9	1.87	+(liquid medium)
Zamora clay loam	Grain	7.2	1.15	?
Botella clay loam	Pasture	6.8	1.86	—
Yolo sandy loam	Lawn	7.8	1.62	900

nitrate and ammonia fractions rather than in the organic fraction. In the last column of Table IV, fixation has been computed on an acre 6 inches deep basis. Actually, discs were at the most 4 cm thick so that the results on an acre basis have been exaggerated. In the case of the photosynthesizing lawn discs, little enhancement of fixation was observed. Under conditions of higher light intensity and more luxuriant photosynthesis, however, there is the possibility that the products of photosynthesis or other metabolic intermediates may be lost to the soil in greater quantities, thereby supporting a more active microflora. Additional experiments are needed for further clarification of these soil-plant interrelations.

Comparison of nitrogen-fixing capacity of different soils and the effect of heavy inoculation of the soil with Azotobacter

Although Yolo is one of the more fertile California soils, and the sample used in the experiments described above contained *Azotobacter*, little nitrogen was fixed even though conditions were thought to be favorable for fixation. A number of other soils tested for comparison were collected directly from the field in Contra Costa County, California. Some of their characteristics are shown in Table V.

Aside from the rather unnatural addition of soluble carbohydrates to the soil, the most effective stimulant of fixation observed so far was the decaying plant material of the inverted sod sections. For the present comparison of various soils, finely chopped grass (tops and roots) was mixed into the soils at the rate of two grams

per 100 grams soil. On the basis of assumptions made earlier this would approximate 20 tons per acre 6 inches deep (fresh weight tops and roots). This material was well dispersed through the soil, however, and therefore not typical of a green manure application nor directly comparable with the inverted sod discs of the previous experiment.

The seven soils, plus an additional three samples (Yolo, Altamont, and Botella) to each of which 1 ml of a dense suspension of *Azotobacter* cells was added, were incubated for 40 days in an atmosphere containing six atom per cent excess N^{15}. At the end of this period the soils were analyzed for ammonium and amide nitrogen, nitrate and organic nitrogen, and the N^{15} content of these fractions was determined. The results are summarized in Table VI. Little fixation took place irrespective of the number of *Azotobacter* cells present or of the soil used. More than 2.5 pounds nitrogen per acre per year was fixed in only two cases, barely significant in an experiment of this nature. Nitrate concentrations reached rather high values at the end of the experiment, but little of this increase was due to fixation.

The effect of nitrate content of the soil on fixation

Nitrate suppression of fixation was studied using glucose as a readily available energy source. One-hundred gram portions of Yolo soil were supplied with various levels of nitrate. Glucose was added at the rate of 10 mg per gram soil (10 tons per acre). Soils were incubated at 21° C for 40 days under an atmosphere of 26 cm N_2^{15} and 15–20 cm O_2.

The results of this experiment are summarized in Table VII. The strong inhibitory effect of nitrate is clearly demonstrated. At low nitrate concentration nitrogen was fixed. However, when the concentration was raised *Azotobacter* still multiplied in large numbers, but little if any nitrogen was fixed.

Relationship of substrate availability to fixation

Since straw is apparently not readily available to *Azotobacter* and must first be broken down by other organisms in the presence of a suitable nitrogen source, soils were incubated with straw alone, with alfalfa added, and with nitrate added. As a control, a sample was incubated with alfalfa alone. The combination of alfalfa and

TABLE VI

Comparison of nitrogen fixation in different soils
100 g soil + 2 g fresh grass and roots; P_w = M.E.; pN_2 = 26 cm; pO_2 = 15–20 cm. Incubated 40 days at 21°C

Soil	*Azotobacter* cells per gram		Ammonia microequivalents per g soil		Nitrate microequivalents per g soil			Organic N fixed microequivalents per g soil	Total N fixed	
	initial	final	final	fixed	initial	final	fixed		microequivalents per g soil	lbs. per acre 6″ deep
Cowell clay	30	5	0.81	0.022	1.25	7.33	0.024	—	0.046	1.3
Rincon clay	300	235	0.48	0.004	0.90	5.23	0.006	—	0.005	0.14
Altamont clay loam . .	—	—	0.79	—	0.65	7.17	—	—	—	—
Altamont, inoculated .	10^6	2×10^8	0.84	0.001	0.65	5.4	0.002	—	0.003	0.084
Manzanita clay loam .	+	—	0.82	—	1.87	9.69	—	—	—	—
Zamora clay loam . .	?	680	0.43	0.004	1.15	6.28	0.006	0.008	0.018	0.50
Botella clay loam . . .	—	—	0.89	0.002	1.86	10.2	0.008	0.069	0.079	2.2
Botella, inoculated . .	10^6	2×10^6	0.66	—	1.86	10.6	—	0.065	0.065	1.8
Yolo sandy loam . . .	900	300	0.47	0.006	1.62	9.8	0.008	0.015	0.029	0.81
Yolo, inoculated . . .	10^6	2×10^8	0.38	0.002	1.62	9.1	—	—	0.002	0.056

TABLE VII

The effect of nitrate content of the soil upon fixation
100 g fresh Yolo soil + 1 g glucose; pH = 7.8; P_w = 27%; pN_2 = 26 cm; pO_2 = 15–20 cm.
Incubated 40 days at 21°C

Nitrate microequivalents per gram soil		*Azotobacter* cells per g soil		Ammonia microequivalents per g soil		Nitrate fixed microequivalents per g soil	Organic N fixed microequivalents per g soil	Total N fixed	
initial	final	initial	final	final	fixed			microequivalents per g soil	lbs. per acre 6″ deep
1.62	0.23	900	9×10^5	0.80	0.017	0.014	1.37	1.40	39
6.62	0.76	900	5.6×10^5	0.56	0.003	0.003	0.044	0.50	14
11.6	1.88	900	7.5×10^4	0.36	0.014	0.022	—	0.036	1.0
21.6	10.9	900	1.0×10^5	0.46	0.005	0.007	0.018	0.029	0.81
41.6	29.6	900	3.6×10^3	0.41	—	—	0.021	0.032	0.9

TABLE VIII

Relationship of substrate type to fixation

100 g Yolo soil; P_w = M.E.; pH = 7.8; pN_2 = 26 cm; pO_2 = 15–20 cm; *Azotobacter*: 900 cells per g; initial nitrate = 1.62 microequivalents per g soil. Incubated 40 days at 21°C

Substrate added per g soil	*Azotobacter* cells per g soil (final)	Ammonia microequivalents per g soil		Nitrate microequivalents per g soil		Organic N fixed microequivalents per g soil	Total N fixed	
		final	fixed	final	fixed		microequivalents per g soil	lbs. per acre 6″ deep
10 mg grass and roots	900	0.40	0.008	6.5	0.013	—	0.021	0.59
20 mg grass and roots	300	0.47	0.006	9.8	0.008	0.015	0.029	0.81
40 mg grass and roots	250	0.50	0.006	14.2	0.014	0.067	0.087	2.4
10 mg straw	120	0.40	0.004	—	—	—	0.004	0.11
10 mg straw and 1 milliequivalent NO_3	200	0.48	0.008	1.36	0.001	—	0.009	0.25
10 mg alfalfa	600	0.65	0.0004	10.3	—	—	0.0004	0.011
10 mg straw and 1 g alfalfa	250	0.52	0.011	3.03	0.008	—	0.019	0.53
10 mg starch	330	0.58	0.002	0.17	—	0.121	0.12	3.5
10 mg glucose	900,000	0.80	0.017	0.23	0.136	1.37	1.40	40

straw was thought to be favorable to fixation because the decomposition products of alfalfa are apparently quite readily available to micro-organisms and may provide a nitrogen source. A few other treatments were also tried as shown in Table VIII.

Significant fixation took place only when glucose was added. The various combinations of straw, alfalfa and nitrate failed to result in any appreciable nitrogen fixation even though with straw alone the nitrate level in the soil was low. Similarly with starch, which would appear to be a good substrate, little nitrogen was fixed.

Except for the soil to which glucose was added, the number of *Azotobacter* cells (or clusters) either remained constant or decreased somewhat.

DISCUSSION

Extensive investigations by Romell[13]) and others[4]) [14]) show that, though the soil atmosphere is relatively immobile and in some finer pores the soil solution may become quite anaerobic, the average soil oxygen tension closely resembles that of the air above it. Therefore, oxygen will probably not be a limiting factor in these soils.

Probably the most important factors limiting fixation under natural conditions are the lack of suitable organic substrate and the presence of nitrate. In many experiments on nitrogen fixation in soil, substrate has been added at a rate inconsistent with that found under natural conditions. In the present experiments attempts were made to add substrates that were qualitatively as well as quantitatively similar to those that might be expected under fallow conditions.

These experiments show clearly that substrates such as straw or starch support little fixation. Other substrates might become more readily accessible to *Azotobacter*, but often their decomposition results in the accumulation of nitrate which suppresses fixation.

The effect of nitrate on nitrogen fixation has been studied by Zoond[20]), Bonazzi[1]) and others who reported a pronounced suppression of fixation above a minimum level. Winters[18]) found that 40–80 mg nitrate-N per liter soil solution stimulated fixation and stated further that "judicious use of nitrogenous

fertilizers does not interfere with nitrogen fixation." His results are surprising, however, since in 2 to 3 weeks he found fixation as high as 5 per cent of the nitrogen originally present in the soil, without any added energy source. Zoond [20] observed that at a concentration of 0.003 per cent nitrogen (amino-, nitrate-, or peptone-N) optimum conditions existed for nitrogen fixation, above that level fixation was depressed. Comparing these investigations with our own data, we may assume that at nitrate concentrations greater than 70 100 mg nitrate-N per liter soil solution (1 to 1.5 microequivalents nitrate per gram soil at $P_w = 20$ per cent) fixation may be suppressed.

Although in 34 of the 50 samples analyzed in these experiments nitrate concentrations were below 1.5 microequivalents per gram soil, in only 2 cases (where glucose or sucrose was added) was more than 40 pounds nitrogen fixed per acre during the experimental period. One soil to which starch had been added showed fixation of 3.5 pounds per acre and an inverted sod disc fixed 12 pounds per acre. The remaining 46 samples fixed less than 4 pounds per acre.

The conclusion which appears inevitable from these data is that under fallow conditions, non-symbiotic aerobic fixation does not exceed a few pounds nitrogen per acre per year. This appears to be a very low value compared to other estimates, especially the careful lysimetric experiments with California soils performed by Chapman and coworkers [3]. These investigators found as much as 48 pounds nitrogen fixed per acre per year under a Sudan-grass — cereal straw rotation. This would suggest that such fixation may have taken place during periods of active photosynthesis, a possibility which is being investigated.

Jensen [5], after an extensive study of Australian wheat soils, concluded:

"There seems to be no foundation for the frequently expressed opinion that soils from arid climates have an extraordinary nitrogen-fixing power and may, through the utilization of crop residues by free living nitrogen-fixing organisms, be permanently under cereal cultivation without depletion of nitrogen. In Australian wheat soils we can normally expect *no gain at all* and only under exceptionally favorable circumstances a fixation corresponding to one-third of the nitrogen requirements of the crops on wheat land worked on the usual wheat-fallow rotation."

Fixation by *Azotobacter* does not necessarily play the major part in

non-symbiotic nitrogen fixation. Olsen [10]) studied decomposing oak and beech leaves (containing 75–90 per cent water) and found that fixation accounted for a 10 per cent increase in nitrogen over the amount originally present. Fixation was not due to *Azotobacter*, since this bacterium could not be demonstrated in the leaves. *Clostridia* were found, however, and more nitrogen was fixed under anaerobic (water saturated) than under aerobic conditions. In agreement with later work by Jensen [7]) Olsen pointed out the slow rate of nitrogen fixation which, after 100 days, was less than 50 per cent complete when beech leaves were used.

Jensen [6]) [7]) and Jensen and Swaby [8]) later demonstrated that various combinations of *Azotobacter* and facultative cellulose decomposing organisms, as well as associations of *Clostridia* and aerobic cellulose decomposers may, after an initial lag, fix nitrogen in cultures. Since, however, a certain degree of anaerobiosis is required for this process, these workers considered it unlikely that this fixation occurs in soils of semi-arid regions. Considering the time lag in fixation, they postulated that this fixation will be significant only in soils which are deficient in oxygen (water saturated) for a considerable length of time.

SUMMARY

1. The fixation of N^{15}-labeled nitrogen in small vessels of California soil under various conditions of pH, substrate level, oxygen tension, and other soil conditions was observed.

2. Nitrate concentrations greater than 1.0–1.5 microequivalents per gram soil were found to suppress nitrogen fixation but not the growth of *Azotobacter*.

3. Large amounts of nitrogen were fixed when soluble organic substrates (*e.g.* glucose or sucrose) were added to the soil.

4. Moderate fixation also resulted from the inversion of a disc of sod.

5. Fixed nitrogen appeared largely in the nitrate and ammonia-amide fractions with that in the nitrate fraction probably representing nitrification of more reduced initial products of fixation.

6. Under conditions of these experiments growing grass did not enhance fixation. At higher light intensities, however, such an enhancement might be observed.

7. The incorporation of grass cuttings, straw or alfalfa meal into the soil caused only a slight increase in fixation.

8. The inoculation of soils with large populations of *Azotobacter* did not result in increased fixation.

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MOLYBDENUM STATUS OF LAMINAE AS DETERMINED BY BIOASSAY AND CHEMICAL METHODS

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The determination of the concentration of molybdenum in molybdenum deficient leaf material is often difficult as the concentration may be well below 0.1 ppm and the quantity of plant material limited. Consequently an efficient bioassay procedure, by means of which minute quantities of molybdenum in leaf digests can be estimated, is desirable for the study of the role of molybdenum in plant nutrition. To evaluate the applicability of such a bioassay method to subsequent and more extensive experiments a comparison was made of the foliar molybdenum concentrations found by a bioassay [2]) and by a chemical [4]) method. Analysis was made of laminae of rubber plants (*Hevea brasiliensis*) which had been grown under varying nutrient regimes. Both methods indicated that sulphur deficient laminae accumulated more than twice as much molybdenum as those of plants supplied with complete nutrient but some disparity existed between the trends shown for a few of the remaining treatments.

EXPERIMENTAL

Seedlings of *Hevea brasiliensis* were grown in sand culture at deficiency or low levels of each of the macronutrient elements and of iron, manganese and boron. Each nutrient contained 0.019 ppm of molybdenum which was supplied as ammonium molybdate ($(NH_4)_6Mo_7O_{24}.4H_2O$); sulphur, when applied, was given in the form of sulphate. Plants of each deficiency treatment exhibited a reduced growth and, generally, visual symptoms; a description of one of

these experiments has been given [1]). In Experiment I seedlings of clone *Tjirandji 1* were grown and the leaf samplings, for the analyses described here, were carried out on 7 and 10 month old plants; for Experiment II, conducted a year later, seedlings of clone *Prang Besar 86* were grown and the samplings carried out on 3 and 4 month old plants. For both experiments leaf samples were taken from each of the two treatment replicate plots, midribs were excised and discarded, and the laminar residue dried and finely powdered before analysis.

Aspergillus niger, strain M was selected as the test organism for the bioassay and the procedure adopted was, with but few modifications, that of Hewitt and Hallas [2]). One hundred milligrams of laminae were acid digested, precisely according to Hewitt and Hallas [2]), made up to 100 ml with water and a 2-ml aliquot taken for the bioassay. The nutrient medium contained twice as much potassium nitrate and half the amount of magnesium sulphate prescribed by Hewitt and Hallas [2]) and the incubation was carried out at 28.5°C. Each sample was bioassayed; for each complete set of replicates a standard molybdenum growth curve was simultaneously prepared. The amount of molybdenum present in the aliquot was obtained by direct reference to the growth, or calibration, curve. The mycelial dry weight range varied from 100 to 800 mg for a corresponding molybdenum range of 0—0.01 μg Mo per 50 ml of nutrient medium.

The chemical estimation of molybdenum in dried laminae was based upon the measurement of the green complex (dissolved in amyl acetate) formed between molybdenum and dithiol (4 methyl-1 : 2 dimercaptobenzene). The procedure adopted was precisely that described by Piper and Beckwith [4]); it necessitated 2–4 grams of lamina for a single estimation. For Experiment I there was usually sufficient material for each replicate sample to be analysed but for Experiment II one analysis was carried out per treatment, on a sample obtained by bulking leaf samples of both replicates, for each sampling.

RESULTS

Both bioassay and chemical methods of analysis clearly showed that sulphur-deficient laminae possessed a higher concentration of molybdenum than those of the complete nutrient plants (Table I). Results from both methods also suggested that a low phosphorus status diminished the molybdenum concentration of the laminae but this effect did not achieve significance.

On the other hand the effect of a mineral deficiency was sometimes contradictory (for example, Experiment II: -N, - B) depending upon whether the results had been obtained by the bioassay or

TABLE I

Molybdenum concentrations of laminae of plants grown under varied mineral status. Results expressed as ppm Mo of dry laminae

Nutrient treatment	Experiment I Clone Tjir. 1 Top and 2nd whorl laminae		Experiment II Clone P.B. 86 2nd and 3rd whorl laminae	
	Bioassay	Chem. anal.	Bioassay	Chem. anal.
Complete nutrient	1.19	1.40	1.18	1.42
—N	1.19	1.52	0.55 *)	1.62
—S	3.51 *)	3.38 *)	3.88 *)	3.38 *)
—P	0.95	1.04	1.02	0.87
—Mg	1.04	1.24	1.15	1.62
—K	0.62 *)	1.10	0.74 *)	1.46
—Ca	1.14	1.12	1.12	1.70
—Fe	1.42	1.76	1.07	1.41
—Mn	1.31	1.56	1.16	1.30
—B	1.42	1.61	1.62 *)	1.28
S.E. of treatment mean	±0.11	±0.12	±0.10	±0.21

*) Differed significantly from complete nutrient value, $P < 0.05$

chemical method. The bioassay procedure also revealed a significant reduction in molybdenum concentration due to potassium deficiency for both experiments; the value obtained by chemical analysis for Experiment I suggested a similar effect but this was not apparent in Experiment II.

Thus it appeared that, for the small range of molybdenum concentration herein investigated, the interpretation of the results could vary according to the method of analysis employed. The chemical method, which was much less tedious and exacting to operate, suffered the disadvantage of requiring a relatively large amount of dried laminae for a single determination. Some of the divergent results obtained by the bioassay method suggested that constituents, other than molybdenum, of the digest aliquot may have interfered with the growth of the fungus and thus produced an apparently lower or higher value for the molybdenum concentration. Such effects seemed to be brought about by potassium-deficient (Experiments I and II) and nitrogen deficient (Experiment II) laminae. The use of digestion blanks with and without added molybdenum, indicated that these effects were probably not caused by residual acids.

There seems to be no alternative to the bioassay procedure for molybdenum estimation when the amount of laminar material available is very small but the results so obtained must be interpreted with caution. While the values obtained by the bioassay procedure may often approximate numerically to those obtained by chemical means the effect of various nutritional treatments may so alter the components of the laminae, as to interfere with the extremely sensitive bioassay procedure and thus produce qualitatively different treatment effects. Such an alteration in laminar composition might also affect any chemical form of estimation but the margin allowed for such interference is usually so large that it has no real importance.

The positive effect of sulphur deficiency on the molybdenum concentration of the laminae possessed a broader significance. It provided complementary support to work carried out by Mulder[3]) and Stout, Meagher, Pearson and Johnson[5]) who showed that sulphate addition decreased molybdenum absorption. It also could be used to discriminate between instances of nitrogen and sulphur deficiencies in *Hevea brasiliensis* which exhibits similar poorly defined yellowish symptoms under deficiency conditions of either element. Analysis of the foliage frequently fails to reveal a significant difference between the nitrogen concentration of nitrogen-deficient laminae and those of complete nutrient plants; similarly for the sulphur concentration of sulphur deficient and complete nutrient laminae (Bolle-Jones[1])). These two deficiencies can, however, be easily resolved on the basis of their laminar molybdenum concentration; nitrogen-deficient laminae are not expected to accumulate molybdenum while a well defined increase may be anticipated in sulphur-deficient laminae as compared with those of normal plants.

SUMMARY

1. Seedlings of *Hevea brasiliensis* were grown in sand culture under conditions of varied mineral status; the laminae were analysed for molybdenum by a bioassay and by a chemical method.

2. Both the bioassay and chemical methods indicated that sulphur-deficient laminae possessed a much higher concentration of molybdenum than those of normal plants.

3. The interpretation of the effects of some of the treatments on the

accumulation of molybdenum in laminae depended on the method of molybdenum estimation; it was suggested that some laminae, even after wet digestion, contained constituents which interfered with the bioassay method of estimation.

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EFFECT OF THE NITROGEN, PHOSPHORUS, POTASSIUM AND MAGNESIUM NUTRITION OF POTATO PLANTS ON THE CONTENT OF FREE AMINO-ACIDS AND ON THE AMINO-ACID COMPOSITION OF THE PROTEIN OF THE TUBERS

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INTRODUCTION

It is a well-known fact that the mineral nutrition of plants may affect the protein and the soluble non-protein nitrogen contents of the tissues. Plants amply supplied with nitrogenous nutrients, for instance, generally have a considerably higher content of protein and in particular a higher content of non-protein nitrogen than those with a poor nitrogen supply. In the case of phosphorus, potassium, and magnesium, deficient plants are usually higher in these nitrogen fractions than normal plants.

In plant-nutrition experiments the number of nitrogenous compounds estimated is often limited; protein and soluble non-protein fractions and, in some cases, the amides asparagine and glutamine, and the amino-acids tyrosine and arginine, are usually determined. Recently developed methods of quantitative chromatography, however, enable a more detailed investigation of the nitrogenous fractions to be made.

Potato tubers have been chosen as the test material in the present investigation for the following reasons: (*a*) they can be stored for a considerable length of time without much alteration in their chemical composition, (*b*) the protein can be obtained in

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a relatively pure state much more easily than in the case of other plant materials, (*c*) a considerable amount of physiological and biochemical data concerning these materials has been collected in this laboratory during the last ten years, (*d*) potato tubers are important from the point of view of human and animal nutrition.

EXPERIMENTAL PROCEDURE

The potato tubers used in the present study were obtained from field experiments in which the plants were grown under widely differing nutritional conditions. For this purpose certified seed potatoes of the varieties Noordeling and Voran were planted on sandy or peaty soils, poor in nitrogen, phosphorus, potassium or magnesium. In some cases the soils used were deficient in more than one nutrient element. The potato plants were dressed with different amounts of the element in which the soil was deficient; the other nutrient elements were mostly supplied in optimum amounts. The tubers were harvested and transported carefully to the laboratory where they were stored in shallow layers in wooden boxes at a temperature varying from 5 to 10°C.

Analytical procedure

Preparation of the sample.

For each analysis longitudinal and transverse slices are cut from 25 tubers in order to obtain a representative sample. These slices are cut up into small pieces and 50 g of the latter are ground in a mortar after the addition of a trace of $NaHSO_3$. The pulp is transferred to a Büchner funnel and washed with water sufficient to give 500 ml of solution. This solution contains the bulk of the protein and the soluble non-protein N-compounds (see Table I). The solution is heated for 5 minutes in a boiling water-bath after the addition of a few drops of acetic acid and a knife point of sodium

TABLE I

Nitrogen contents of filtrate (divided into protein-N and soluble non-protein N) and of residue of ground potato tubers (mg per 10 mg fresh weight)

Protein-N	Soluble non-protein N	Residue-N
32.1	29.1	2.1
33.8	34.7	3.1
35.1	27.3	1.6
29.4	20.9	3.7
23.4	23.3	2.7
20.2	20.7	3.3
19.0	21.1	2.1
18.3	23.8	2.3

chloride. The protein which is precipitated is then separated by centrifugation. The precipitate is washed twice with a very dilute solution of acetic acid and three times with 95 per cent ethanol. The washed protein is transferred to dishes, dried at 37 C and ground in a mortar. It contains approximately 14.5 per cent nitrogen (see Table II), corresponding to a purity of 90%, based on protein of 16% N.

TABLE II

Manurial treatment	N-content of potato protein	
	N %	Purity of protein *)
low N	14.65	91.6
high N	14.8	92.5
low P	14.0	87.5
high P	13.85	86.6
low K	14.8	92.5
high K	14.6	91.3

*) Based on protein of 16% N.

The filtrate which contains the amino-acids, the amides and the peptides is concentrated *in vacuo* at a temperature not exceeding 40°C, and made up to 25 ml.

To obtain a larger amount of protein it was found more convenient to use a separate sample of 200 g tuber tissue. This was macerated in a Waring blendor and treated as described above.

Estimation of protein and soluble non-protein nitrogen.

Estimations of the nitrogen contained in these fractions are carried out on separate samples of 10 g tuber tissue. These are ground and separated from starch and other cell residues by filtration on a Büchner funnel. The protein of the filtrate is precipitated as described above. The nitrogen of the latter plus that of the cell residues is calculated as protein nitrogen, that of the filtrate from the heat coagulum as soluble non-protein nitrogen.

Hydrolysis of the protein.

The protein is hydrolysed in 6*N* HCl for 16–18 hours (500 mg of protein in 25 ml redistilled HCl). After hydrolysis the hydrochloric acid is removed by repeated distillation *in vacuo* (5–6 times). The HCl should be removed as completely as possible to prevent the formation of interfering amounts of NaCl when this solution is brought up to pH 6.0 with NaOH. After the addition of NaOH the solution is filtered and made up to 50 ml.

The protein hydrolysates and the protein-free solutions were analysed for amino-acids by the quantitative paper chromatographic method of Thompson and Steward [12] [13]. This method will be described in some detail.

Quantitative paper chromatography of amino-acids.

The solution to be analysed should contain approximately 4 mg of amino-acid-N per ml. For a complete analysis of the amino-acids two different dilutions of this solution are used containing (*a*) 50 and (*b*) 100–200 μg of N per 0.05 ml, respectively. The dilute solution (*a*) is used for those amino-acids which, in large concentrations, may overlap (aspartic acid and glutamic acid, serine and glycine, alanine and threonine). Solution (*b*) is used for all other amino-acids. A second aliquot of 0.05 ml of (*b*) is used for the S-containing amino-acids (see below). 0.1 ml of (*b*) is used for the estimation of those amino-acids which occur in very small amounts or which give a weak colour with ninhydrin (lysine, tyrosine, proline).

The sample is applied to a sheet of filter paper at a point 8 cm from top and side of the paper and is confined to a circle 1–1.5 cm in diameter (drops of 0.01 ml are used). To support the paper a watch glass is placed below the circle during the operation. To promote the drying of the drops, heat from an electric lamp is used; this may be done as long as the spot is moist. After drying, mostly for 5 minutes, the paper is transferred to the cabinet in which the chromatography (descending method) is carried out. A 2-directional procedure was used first with phenol-water and then with collidine-lutidine-water as the moving phases. The cabinet used contains three troughs (see Fig. 55b in [15]); in each trough two sheets of filter paper may be placed. The papers are held in the troughs by a heavy glass rod in such a way that the sample lies approximately 3.5 cm beyond the edge of the trough. Dishes with phenol-water (2 : 3) are placed at the bottom of the cabinet to allow the papers and the atmosphere to equilibrate with this solvent before adding the latter to the troughs (approximately 8 hours).

80 ml of phenol-water (3 : 1, pH 5–5.5) are then added to the troughs through a small hole in the top of the cabinet which is closed by a stopper. The phenol solution travels downwards through the paper, carrying the different amino-acids at different rates. When the solvent has moved for approximately 45 cm, *i.e.* when it has reached a distance of approximately 1–2 cm from the lower edge (36 hours at 20°C) the paper is removed from the cabinet and allowed to dry at room temperature in an air stream for about 20 hours. A margin of 2 cm containing the phenol front is cut from the paper. The paper is then transferred to a second cabinet in which a similar procedure is carried out with collidine-lutidine-water (1 : 3 : 3, pH 8.0) in a direction at right angles to the first. As with the phenol treatment the second cabinet contains dishes with the solvent to ensure equilibrium between paper and atmosphere. After equilibrium has been reached (4–6 hours) 80 ml of the collidine-lutidine-water mixture are added to the troughs and the second phase of the chromatography is begun. After 20–24 h at 20°C when the solvent has moved for approximately 35 cm the procedure is stopped and the paper is again dried in an air stream (about 16 hours).

Development of the chromatogram: reaction of the

amino-acids on the paper with ninhydrin. For this purpose the chromatogram is sprayed evenly with a solution of 1% ninhydrin in 95% ethanol containing 2% of a collidine-lutidine mixture (1 : 3) (*ca* 30–35 ml per sheet of filter paper 35 by 45 cm *). A hand sprayer activated by compressed CO_2 is used. An excess of the solvent may cause the coloured spots to spread and should therefore be avoided.

The sheets are then transferred to the colour development cabinet which is a modification of the apparatus of T h o m p s o n *et al.* (see Fig. 3 in [12]). It consists of a tank 54 by 34 by 60 cm in which the chromatograms are heated under ethanol-saturated anaerobic conditions. The bottom of the tank contains a layer of ethanol. Three perforated pipes connected to a metal cylinder containing carbon dioxide are immersed in this layer so that a flow of ethanol-saturated CO_2 passes across the paper sheets. The tank has accomodation for three sheets. A perforated shield above the surface of the ethanol prevents splashing of the papers. The papers containing the amino-acids are hung vertically in the tank with the initial spot at the lower end. In order to keep the atmosphere saturated with ethanol two filter papers hang down along the walls of the tank from shallow troughs at the top. These filter papers dip into the ethanol at the bottom of the tank; the troughs also contain ethanol. The tank is contained in an outer cabinet with a constant-temperature control.

Under these conditions an optimum colour development was found to take place within 20–25 minutes at 60°C. The sheets are then dried for 10 minutes in air. No oxidation of the coloured compound was found to occur during drying.

The coloured spots are cut out and extracted with 10 ml 50% ethanol (by volume) for 30 minutes after being cut into pieces of 0.5 by 2 cm. The intensity of the extracted blue (or yellow-brown) compound is measured in a colorimeter at 570 mμ (blue compound) or 330 mμ (yellow-brown compound).

From each sheet blanks of similar size are cut out and also extracted. When the extractions are made immediately after completion of the colour reaction the blank value is low; it increases after 1–2 hours.

R e m a r k s.

P a p e r. Whatman no. 1 filter paper, "for chromatography", 45 by 55 cm, was used throughout the investigation. The paper was not washed before use.

P u r i f i c a t i o n o f s o l v e n t s. Phenol as well as collidine and lutidine were redistilled before use. The latter two products were purchased from N.V. Utrechtse Asphaltfabriek (The Hague) and distilled at 168°C (collidine) and between 153–157°C (lutidine).

S t a n d a r d c u r v e s. As different amino-acids on paper react in different ways with ninhydrin, standard curves for each amino-acid have to be made. For this purpose 20, 30, 40, 50 and 60 μg respectively of each

*) In addition to the removal of margins of 2 cm containing the phenol and collidine-lutidine fronts, respectively, strips of 8 cm are cut from the opposite sides of the sheets before treatment with ninhydrin.

amino-acid are chromatographed and their colour estimated as described above. Two standard solutions are prepared, one containing aspartic acid, glycine and threonine and the other glutamic acid, serine and alanine. The remaining amino-acids may be in either solution. The pH of the standard solutions is adjusted to pH 6.0. The standard solutions are analysed in three or four replicates.

Internal standard. As a check on the reproducibility of the values, a so-called internal standard is included in each case. For this purpose 40 μg of alanine are placed 13 cm from the top and side of each sheet used. After chromatography it takes a position which is not occupied by other amino-acids. In the case of H_2O_2-treated samples it can not be used in this position as it then overlaps the position of methionine sulphone.

When the internal standard deviates considerably from its standard value the sheet is discarded. Corrections are not made.

Sulphur-containing amino-acids. The sulphur-containing amino-acids, methionine, cystine and related compounds have to be oxidized to methionine sulphone and cysteic acid respectively in order to obtain distinct spots on the chromatogram. This can be done by adding 0.02 ml

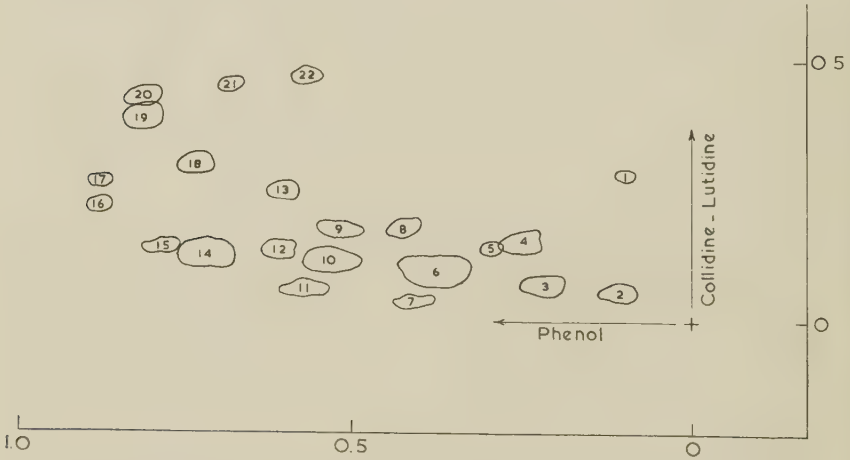

Fig. 1. Two-dimensional chromatogram, showing separation of the amino-acids in the protein and the non-protein fraction of the potato tuber.

1. Cystein + cysteine (after oxidation)
2. Aspartic acid
3. Glutamic acid
4. Serine
5. Glycine
6. Asparagine
7. Lysine
8. Threonine
9. Alanine
10. Glutamine
11. Arginine
12. Histidine
13. Methionine (after oxidation)
14. γ-Aminobutyric acid
15. Methionine sulphoxide
16. Proline
17. Pipecolic acid
18. Valine
19. Leucines
20. Phenylalanine
21. Tryptophane
22. Tyrosine

of 0.02 *M* ammonium molybdate and 0.1 ml of 30% H_2O_2 to 0.1 ml of the amino-acid solution in a watch glass. After drying, the residue is dissolved in water and transferred to the paper.

Ammonia and cyanide were not used in the cabinet during the phenol treatment.

Position of amino-acids on the chromatogram (see Fig. 1). The following amino-acids give distinct spots on the chromatogram: glycine, alanine, serine, cystine (after oxidation to cysteic acid), threonine, valine, methionine (after oxidation to methionine sulphone), the leucines, tyrosine, proline, aspartic acid, glutamic acid, arginine, lysine, γ-aminobutyric acid, asparagine and glutamine. An unknown spot was always present on the chromatogram of the soluble fractions. After most analyses had been performed this spot was found to be brought about by pipecolic acid. Leucine and isoleucine occupy the same position so that they are determined together. Glycine and asparagine more or less overlap so that their separate determination is difficult when both occur in considerable amounts. In the present investigation this point was not very important, since glycine occurs only as traces in the soluble non-protein fraction in which asparagine is an important constituent, while in the protein hydrolysate asparagine is absent. When glycine and asparagine occur in the same sample, hydrolysis of the asparagine to aspartic acid may be used to allow for the determination of both. In this case the sample has to be chromatographed before and after hydrolysis. The increase in aspartic acid upon hydrolysis gives the value for asparagine. To decide whether a certain spot on the chromatogram represents a certain amino acid, a second aliquot of the same sample, to which an additional amount of the expected amino acid has been added, is chromatographed. Phenylalanine, tryptophane and histidine cannot be determined by this chromatographic method. For their estimation see below.

Accuracy of the method. The amino-acids may be determined with an accuracy of 12% or less. Table III gives the recovery values for different amino-acids added to a potato extract. The amino-acids were added in amounts of 40 μg.

Phenylalanine.

Phenylalanine is estimated according to the method of Kapeller–Adler[6]). This is based on the oxidation and nitration of the amino-acid to 3,4-dinitrobenzoic acid which after reduction with NH_2OH gives a

TABLE III

Recovery of amino-acids added to a potato extract

Amino-acid	Recovery (%)	Amino-acid	Recovery (%)
Alanine	96	Glutamic acid	106
Valine	98	Arginine	105
Leucine	102	γ-aminobutyric acid	96
Tyrosine	94	Asparagine	103
Aspartic acid	97	Glutamine	106

purple-blue colour with ammonia, presumably due to the formation of the ammonium salt of *o*-diaci-dihydro-dinitrobenzoic acid.

Reagents. (*a*) Nitration mixture: 20 g KNO_3 are dissolved in 100 ml concentrated H_2SO_4. (*b*) 30% $NH_2OH.HCl$. (*c*) 25% NH_4OH.

Method. An aliquot of an amino-acid solution is evaporated to dryness. 2 ml of (*a*) are added and the nitration is carried out by warming on the steam bath for 20 minutes. The residue is transferred with 9 ml of H_2O to a volumetric flask of 25 ml. The solution is cooled in an ice bath, and 2.5 ml of (*b*), also cooled in the ice bath, are added. After 1 minute the solution is diluted to the mark with ice-cooled ammonia (*c*). The mixture is allowed to stand for 45 minutes at room temperature and then the colour intensity is read at 560 mμ. For each sample a blank value obtained without the addition of $NH_2OH.HCl$ is measured.

Tyrosine and histidine give a yellow-brown colour which does not interfere at 560 mμ. The method is sensitive to 100 μg of phenylalanine.

Tryptophane.

Tryptophane is estimated according to the method of Brummer [2]) (see also Groot [3])). This is based on the formation of a red-violet colour with vanillin in a strongly acid solution. The method is used for protein hydrolysates only, as unknown substances in the potato extract interfere with the determination of this amino-acid.

Reagents. (*a*) 1/75 *M* solution of vanillin in H_2O. (*b*) 96% H_2SO_4 (*pro analysi*). (*c*) 1/450 *M* Na_2S. Prepared by dissolving an excess of Na_2S in H_2O; the solution is titrated with iodine and then diluted with water to give the desired concentration.

Method. 1 ml of amino-acid solution, 1 ml of (*c*) and 0.1 of (*a*) are added to a test tube and cooled in a NaCl-ice bath. Then 3 ml of (*b*) are slowly added; care is taken that the solution remains as cold as possible; it is shaken continuously during the addition of the H_2SO_4. The colour is allowed to develop at room temperature for 4½ h after which the flask is placed on a boiling water bath for 3–4 minutes. The colour intensity is measured at 605 mμ. The method is sensitive to 5 μg of tryptophane.

Histidine.

Histidine is determined by the diazo reaction of Pauly: formation by histidine of a yellow-red compound with freshly diazotized sulphanilic acid (Block and Bolling [1])). Since tyrosine gives the same reaction, this amino-acid has to be removed before histidine can be determined. Use is made of the fact that tyrosine may be oxidized by 0.1 *N* $KMnO_4$ in an acid solution, without histidine being affected. The amount of $KMnO_4$ required for complete removal of tyrosine from a protein-free potato extract was found to be 5 to 6 times higher than was needed for elimination of an equal amount of tyrosine in the absence of other substances. Upon neutralization of the $KMnO_4$-treated sample a yellow-brown colour, presumably due to MnO_2, is formed which interferes with the histidine determination. Therefore the neutralization should be stopped when a faint yellow colour of the solution is observed.

The amount of diazo reagent required for determination of histidine in potato extracts is generally considerably greater than that needed for histidine only. This is presumably due to the fact that in addition to tyrosine, potato extract contains an unknown compound which reacts with the diazo reagent. When this unknown compound is present in large amounts, it may interfere with the histidine determination. This may be prevented by using a smaller aliquot of the extract. To check the absence of this interference two different amounts of the extract are analysed. In addition the recovery of added histidine has to be checked.

Reagents. (*a*) 4.5 g of sulphanilic acid and 45 ml of 37% HCl in 500 ml H_2O. (*b*) 25 g $NaNO_2$ in 500 ml H_2O. (*c*) 5.5 g anhydrous Na_2CO_3 in 500 ml H_2O. (*d*) Solution of histidine-monohydrochloride 1 : 10000. The diazo reagent is prepared by adding 1.5 ml of (*a*) to 1.5 ml of (*b*) in a 50-ml volumetric flask kept in an ice bath. After 5 minutes cooling 6 ml of (*b*) are again added and after a further 5 minutes cooling the solution is diluted to 50 ml. The solution may be kept for 1 day when cooled in ice.

Method. To 15 ml of the protein-free amino-acid solution are added 1 ml of 30% H_2SO_4 and 3 ml of 0.1 *N* $KMnO_4$. The mixture is neutralized with 10% NaOH until it takes on a light yellow colour and then filtered through a dry filter paper. 0.5 and 1 ml of the filtrate are used for the histidine determination which is carried out as follows. 5 ml of (*c*) are pipetted into a 25-ml volumetric flask. To this solution sufficient H_2O is added so that the total volume of added water and aliquot of histidine solution is 2 ml. Subsequently 2 ml of the diazo reagent are added, immediately followed by the histidine-containing solution. When the yellow-orange colour is formed a second portion of 2 ml reagent is added. After 6 minutes incubation in a water bath at 15°C maximum colour intensity is reached. The latter is measured at 604 mμ. The method is sensitive to 10 μg of histidine.

RESULTS

a) Comparison of the amino-acid composition of the protein and the soluble non-protein fractions

Tables V to X contain the amino-acid analyses of the protein and the soluble non-protein fractions of potato tubers grown under widely differing nutritional conditions. In each case a comparison was made between tubers from plants highly deficient in one of the major nutrient elements and those from plants grown on the same field with an ample supply of this element.

In Tables V, VII, VIII and IX the amino-acid values for both protein and soluble non-protein fractions have been recorded; in Tables VI and X those of the non-protein fractions only are given. In the case of protein the figures have been calculated as g amino-acid per 100 g of hydrolysed protein (16 g N) and as

TABLE IV

Yield data and protein and soluble non-protein N-contents of the potato tubers used for the amino-acid analyses

Exp., year, variety	Manurial treatment *) (kg per ha)	Yield, q per ha (1 q = 100 kg)	mg N per 10 g dry weight	
			Protein fraction	Soluble non-protein fraction
1388, 1952, Noordeling	40 N	103	78.7	32.8
	160 N	183	107.4	66.6
1300, 1952, Noordeling	0 P_2O_5	56	113.6	79.7
	300 P_2O_5	249	102.1	73.1
1472, 1954, Noordeling	0 P_2O_5	44	85.5	84.4
	300 P_2O_5	290	62.5	44.8
1299, 1952, Noordeling	0 K_2O	108	106.4	106.9
	200 K_2O	290	93.4	86.4
1472, 1954, Noordeling	0 K_2O	83	86.3	92.9
	400 K_2O	290	65.6	46.7
1220, 1951, Voran	5 MgO	161	79.8	90.6
	160 MgO	383	63.6	56.3
1466, 1953, Noordeling	400 K, 150 N	299	99.8	69.7
,, ,, Voran	400 K, 150 N	455	54.7	54.6

*) N was supplied as ammonium nitrate limestone, P as superphosphate, K as potassium sulphate, and Mg as magnesium sulphate.

amino-acid nitrogen as a percentage of the sum of estimated amino-acid nitrogen (1st and 2nd column of each sample respectively). The values for the free amino-acids are similarly given as g amino-acid per 16 g animo-acid-N (3rd and 4th column of each sample) and as amino-acid nitrogen as a percentage of the total estimated amino-acid nitrogen (5th and 6th column). In the case of free amino-acids, values are given both including and excluding the amides.

When a comparison is made between the content of amino-acids occurring in the free state and that of amino-acids present in the protein, it will be seen that no correlation exists. In the soluble non-protein fraction the nitrogen of the amides constitutes more than 60% and sometimes more than 70% of the total amino-acid nitrogen. In the case of protein, at a maximum, 20% of the total amino-acid nitrogen could be combined as amides, assuming that all the ammonia in the hydrolysate came from these compounds.

When the amino-N of the amides is added to the amino-N of aspartic acid and glutamic acid respectively, it will be seen that

TABLE V

Composition of the protein and the soluble non-protein fractions of potato tubers grown at two nitrogen levels, stored for 4 months (Exp. 1388, 1952, *var.* Noordeling)

Amino compound	Low-N potatoes						High-N potatoes					
	Protein fraction *)		Soluble non-protein fraction *)				Protein fraction *)		Soluble non-protein fraction *)			
	g am.-acid per 100 g protein (16 g N)	N as % of sum of est. am.-acid-N (incl. NH_3)	g am.-acid per 16 g am.-acid-N		N as % of sum of est. am.-acid-N		g am.-acid per 100 g protein (16 g N)	N as % of sum of est. am.-acid-N (incl. NH_3)	g am.-acid per 16 g am.-acid-N		N as % of sum of est. am.-acid-N	
			incl. the amides	excl. the amides	incl. the amides	excl. the amides			incl. the amides	excl. the amides	incl. the amides	excl. the amides
Glycine	5.9	7.5	tr	tr	tr	tr	6.0	7.6	tr	tr	tr	tr
Alanine	5.1	5.4	1.0	2.3	1.0	2.3	5.0	5.4	0.6	2.2	0.6	2.2
Serine	6.8	6.2	1.0	2.3	0.8	2.0	7.2	6.6	0.6	2.2	0.5	1.8
Cystine + cysteine	1.1	0.7	0.7	1.6	0.4	0.9	1.3	0.8	0.3	1.1	0.2	0.6
Threonine	6.5	5.2	1.3	2.9	0.9	2.2	6.5	5.2	0.6	2.2	0.5	1.6
Valine	6.9	5.7	2.8	6.3	2.1	5.0	6.5	5.4	1.7	6.0	1.3	4.5
Methionine + methionine sulphoxide	2.8	1.7	1.9	4.3	1.1	2.6	3.1	2.0	1.0	3.3	0.6	2.0
Leucines	16.2	11.8	3.3	7.5	2.2	5.1	16.3	11.9	2.2	7.5	1.4	5.0
Phenylalanine	5.0	2.9	—	—	—	—	4.9	2.8				—
Tyrosine	4.2	2.2	2.1	4.8	1.0	2.4	4.6	2.4	1.3	4.5	0.6	2.2
Proline	3.8	3.2	tr	tr	tr	tr	3.8	3.2	tr	tr	tr	tr
Tryptophane	1.8	1.6	—	—	—	—	2.0	1.9				
Aspartic acid	10.5	7.5	5.9	13.7	4.0	9.3	10.5	7.5	4.1	14.2	2.7	9.3
Glutamic acid	9.9	6.4	20.6	47.4	12.4	29.3	9.8	6.3	10.3	35.3	6.1	21.1
Arginine	5.8	12.7	5.4	12.4	11.0	25.9	5.5	12.1	4.8	16.5	9.6	33.2
Lysine	4.2	5.4	tr	tr	tr	tr	4.0	5.2	tr	tr	tr	tr
Histidine	2.5	4.6	1.0	2.3	1.7	4.0	2.6	4.8	0.6	1.9	0.9	3.2
γ-Aminobutyric acid	0.0	0.0	4.2	9.7	3.6	8.5	0.0	0.0	4.6	15.8	3.9	13.4
Asparagine	—	—	34.6	—	46.4	—	—	—	41.4	—	54.9	—
Glutamine	—	—	9.1	—	11.1	—	—	—	13.3	—	15.9	—
Ammonia	1.7	9.3	—	—	—	—	1.6	9.0				

*) 91.9% and 76.2% of the Kjeldahl nitrogen of the protein and the soluble non-protein fractions of the low-N potatoes are accounted for by the amino-acid analyses of these fractions. For the high-N potatoes these values are 91.6% and 81.8%, respectively.

aspartic acid occurs even more abundantly than glutamic acid in the non-protein fraction.

The ratio asparagine-N : glutamine-N varies from 4.5 : 1 to 2 : 1. This ratio apparently is not only affected by the mineral nutrition of the potato plants but also by climatic conditions and variety.

TABLE VI

Composition of the soluble non-protein fraction of potato tubers grown at two phosphorus levels, stored for 6 months (Exp. 1300, 1952, *var.* Noordeling)

Amino compound	Low-P potatoes				High-P potatoes			
	Soluble non-protein fraction				Soluble non-protein fraction			
	g am.-acid per 16 g am.-acid-N *)		N as % of sum of est. am.-acid-N *)		g am.-acid per 16 g am.-acid-N *)		N as % of sum of est. am.-acid-N *)	
	incl. the amides	excl. the amides	incl. the amides	excl. the amides	incl. the amides	excl. the amides	incl. the amides	excl. the amides
Glycine	tr	tr	tr	tr	tr	tr	tr	tr
Alanine	0.6	2.1	0.6	2.1	0.6	2.0	0.6	2.0
Serine	1.3	4.8	1.1	4.1	1.1	3.7	0.9	3.1
Cystine + cysteine	0.7	2.5	0.4	1.4	0.6	2.0	0.3	1.1
Threonine	1.0	3.8	0.8	2.8	1.0	3.6	0.8	2.7
Valine	1.5	5.4	1.1	4.1	2.2	7.4	1.6	5.5
Methionine + methionine sulphoxide	0.6	2.1	0.3	1.3	0.5	1.8	0.3	1.0
Leucines	1.7	6.1	1.1	4.1	1.6	5.3	1.0	3.6
Phenylalanine	1.5	5.6	0.8	3.0	1.6	5.3	0.8	2.8
Tyrosine	1.7	6.2	0.8	3.0	2.2	7.5	1.1	3.6
Proline	0.8	2.8	0.6	2.2	1.0	3.4	0.8	2.6
Tryptophane	—	—	—	—	—	—	—	—
Aspartic acid	4.0	14.8	2.6	9.7	4.4	15.0	2.9	9.9
Glutamic acid	9.4	34.7	5.4	20.1	7.0	24.1	4.1	13.9
Arginine	4.0	14.8	7.2	26.4	3.5	11.9	6.2	21.3
Lysine	tr	tr	tr	tr	tr	tr	tr	tr
Histidine	1.6	5.7	2.6	9.7	1.9	6.6	3.3	11.2
γ-Aminobutyric acid	2.0	7.3	1.7	6.2	5.4	18.4	4.6	15.6
Asparagine	44.9	—	59.6	—	38.8	—	51.6	—
Glutamine	11.1	—	13.3	—	16.0	—	19.2	—
Ammonia	—	—	—	—	—	—	—	—

*) 80.9% of the Kjeldahl nitrogen of the soluble non-protein fraction of the low-P potatoes is accounted for by the amino-acid analyses of this fraction. For the high-P potatoes this value is 82.0%.

When the amides are excluded, glutamic acid, arginine, and γ-aminobutyric acid account for more than 50% of the amino-acid nitrogen of the soluble non-protein fraction. On a weight basis glutamic acid is generally by far the most important amino-acid of this fraction followed by γ-aminobutyric acid in the second and arginine in the third place. On a nitrogen basis arginine is generally the most important amino-acid, owing to its high N-content.

In contrast to the non-protein fraction, γ-aminobutyric acid

TABLE VII

Composition of the protein and the soluble non-protein fractions of potato tubers grown at two phosphorus levels, stored for 1 month (Exp. 1472, 1954, *var.* Noordeling)

Amino compound	Low-P potatoes						High-P potatoes					
	Protein fraction *)		Soluble non-protein fraction *)				Protein fraction *)		Soluble non-protein fraction *)			
	g am.-acid per 100 g protein (16 g N)	N as % of sum of est. am.-acid-N (incl. NH_3)	g am.-acid per 16 g am.-acid-N		N as % of sum of est. am.-acid-N		g am.-acid per 100 g protein (16 g N)	N as % of sum of est. am.-acid-N (incl. NH_3)	g am.-acid per 16 g am.-acid-N		N as % of sum of est. am.-acid-N	
			incl. the amides	excl. the amides	incl. the amides	excl. the amides			incl. the amides	excl. the amides	incl. the amides	excl. the amides
Glycine	5.4	7.6	0.5	1.9	0.6	2.3	5.8	8.0	1.0	2.8	1.1	3.4
Alanine	4.1	4.8	0.6	2.5	0.6	2.4	4.3	5.0	0.7	2.1	0.7	2.1
Serine	5.8	5.8	0.8	3.1	0.7	2.6	6.0	5.9	1.3	3.9	1.1	3.4
Cystine + cysteine	0.6	0.4	0.2	0.7	0.1	0.4	0.7	0.5	0.3	0.7	0.1	0.4
Threonine	6.0	5.3	0.8	3.3	0.6	2.4	5.8	5.1	1.0	3.0	0.8	2.3
Valine	7.3	6.7	2.2	8.8	1.7	6.6	7.6	6.7	2.8	8.3	2.1	6.2
Methionine + methionine sulphoxide	1.9	1.3	1.2	4.8	0.7	2.8	2.0	1.4	2.0	5.7	1.2	3.5
Leucines	17.0	13.8	1.7	6.7	1.3	4.5	17.9	14.1	2.1	6.2	1.4	4.1
Phenylalanine	5.0	3.3	1.9	7.6	1.0	4.0	5.3	3.3	1.8	5.3	1.0	2.8
Tyrosine	4.5	2.7	1.2	4.6	0.6	2.2	4.6	2.6	2.7	7.8	1.3	3.7
Proline	3.6	3.3	tr	tr	tr	tr	3.6	3.2	tr	tr	tr	tr
Tryptophane	1.8	1.8	—	—	—	—	1.9	1.9	—	—	—	—
Aspartic acid	8.0	6.4	2.2	8.6	1.4	5.6	8.1	6.3	2.7	7.8	1.8	5.1
Glutamic acid	9.5	6.9	7.6	30.3	4.5	18.0	10.0	7.0	10.9	31.8	6.5	19.0
Arginine	3.2	7.8	3.4	13.4	6.8	27.0	3.4	8.0	2.9	8.6	5.9	17.1
Lysine	3.7	5.3	tr	tr	tr	tr	3.9	5.5	tr	tr	tr	tr
Histidine	3.1	6.4	0.8	3.2	1.4	5.4	3.0	6.0	1.3	3.8	2.2	6.4
γ-Aminobutyric acid	0.0	0.0	4.1	16.4	3.5	14.0	0.0	0.0	8.6	25.0	7.3	21.2
Asparagine	—	—	37.3	—	52.4	—	—	—	32.3	—	43.1	—
Glutamine	—	—	18.6	—	22.3	—	—	—	18.7	—	22.4	—
Ammonia	1.7	10.3	—	—	—	—	1.6	9.5	—	—	—	—

*) 82.5% and 70.0% of the Kjeldahl nitrogen of the protein and the soluble non-protein fractions of the low-P potatoes are accounted for by the amino-acid analyses of these fractions. For the high-P potatoes these values are 84.8% and 71.5%, respectively.

does not occur in the protein. Arginine accounts for approximately 10 per cent of the protein-N, glutamic acid for about 6 per cent. A number of amino-acids, however, occur much more abundantly in the protein than in the free state. These are glycine, alanine, serine, threonine, the leucines, proline and lysine.

b) Effect of the mineral nutrition on the amino-acid composition of the protein.

Although the mineral nutrition of the potato plants in this investigation had a pronounced effect on the yield and on the protein content of the tubers (see Table IV) no effect on the amino-

TABLE VIII

Composition of the protein and the soluble non-protein fractions of potato tubers grown at two potassium levels, stored for 3 months (Exp. 1299, 1952, *var.* Noordeling)

Amino compound	Low-K potatoes						High-K potatoes					
	Protein fraction *)		Soluble non-protein fraction *)				Protein fraction *)		Soluble non-protein fraction *)			
	g am.-acid per 100 g protein (16 g N)	N as % of sum of est. am.-acid-N (incl. NH_3)	g am.-acid per 16 g am.-acid-N		N as % of sum of est. am.-acid-N		g am.-acid per 100 g protein (16 g N)	N as % of sum of est. am.-acid-N (incl. NH_3)	g am.-acid per 16 g am.-acid-N		N as % of sum of est. am.-acid-N	
			incl. the amides	excl. the amides	incl. the amides	excl. the amides			incl. the amides	excl. the amides	incl. the amides	excl. the amides
Glycine	5.3	7.0	tr	tr	tr	tr	5.4	6.9	tr	tr	tr	tr
Alanine	5.9	6.6	0.6	1.7	0.5	1.7	6.0	6.6	0.6	1.8	0.6	1.7
Serine	6.6	6.2	1.3	4.1	1.1	3.4	6.0	5.6	1.5	4.6	1.3	3.8
Cystine + cysteine	1.0	0.6	0.4	1.3	0.3	1.0	0.7	0.4	0.5	1.6	0.3	0.8
Threonine	6.7	5.6	1.2	3.7	0.9	2.7	6.5	5.3	1.3	3.8	1.0	2.8
Valine	5.9	5.0	3.0	9.2	2.3	6.9	6.2	5.1	2.6	7.6	1.9	5.7
Methionine + methionine sulphoxide	2.5	1.7	1.5	4.5	0.9	2.7	2.3	1.4	1.7	4.9	1.0	2.9
Leucines	15.3	11.6	1.9	5.9	1.3	3.9	15.2	11.3	1.7	5.1	1.1	3.4
Phenylalanine	5.5	3.3	2.3	6.9	1.2	3.7	5.4	3.2	1.4	4.2	0.8	2.2
Tyrosine	5.2	2.8	5.2	15.9	2.5	7.7	5.0	2.7	3.3	9.7	1.6	4.7
Proline	4.3	3.7	tr	tr	tr	tr	5.2	4.4	tr	tr	tr	tr
Tryptophane	1.9	1.8	—	—	—	—	1.8	1.7	—	—	—	—
Aspartic acid	10.3	7.7	2.8	8.4	1.8	5.6	10.5	7.7	3.9	11.4	2.5	7.5
Glutamic acid	9.4	6.4	5.8	17.8	3.5	10.6	9.9	6.6	8.6	25.3	5.1	15.1
Arginine	4.2	9.5	4.6	14.0	9.2	28.0	4.4	9.8	5.2	15.3	10.4	30.7
Lysine	4.6	6.2	0.7	2.2	0.9	2.7	5.0	6.7	0.8	2.4	1.0	2.9
Histidine	2.6	5.0	0.6	1.8	1.0	3.1	2.4	4.6	0.6	1.9	1.1	3.1
γ-Aminobutyric acid	0.0	0.0	6.4	19.5	5.4	16.6	0.0	0.0	5.2	15.5	4.5	13.1
Asparagine	—	—	37.9	—	50.3	—	—	—	38.6	—	51.2	—
Glutamine	—	—	14.1	—	16.8	—	—	—	12.3	—	14.8	—
Ammonia	1.6	9.3	—	—	—	—	1.8	10.0	—	—	—	—

*) 88.2% and 87.8% of the Kjeldahl nitrogen of the protein and the soluble non-protein fractions of the low-K potatoes are accounted for by the amino-acid analyses of these fractions. For the high-K potatoes these values are 89.9% and 89.1%, respectively.

acid composition of this protein was observed (see Tables V, VII, VIII and IX). This was found to be true in all three cases, nitrogen, potassium and phosphorus. Since the composition of the soluble non-protein fraction varied considerably under the influence of the mineral nutrition, this result once more demonstrates the lack of correlation between the amino-acid composition of the protein and that of the soluble non-protein fractions.

When a comparison is made between the proteins of tubers grown on the same field with different manurial treatments and those of tubers grown on different fields and in different years, it will be seen that the agreement in amino-acid composition in the former case is more complete than in the latter. Although it is possible that small differences in protein composition of potato tubers may occur under the influence of varying climatic conditions, it is also possible that small differences in the analytical technique have been responsible for the observed differences.

c) *Effect of the mineral nutrition on the amino-acid composition of the soluble non-protein fraction.*

The amino-acid composition of the non-protein fraction was found to be much less constant than that of the protein. Tables V, VII, VIII and IX contain the results for samples in which both protein and non-protein fractions had been analysed. In Table VI and X the results for a few further samples have been recorded in which non-protein nitrogen only was investigated.

Nitrogen. Tubers from nitrogen-deficient potato plants were considerably poorer in both protein and soluble non-protein nitrogen than those from plants dressed amply with ammonium nitrate. The non-protein nitrogen content was more strongly affected by the nitrogen dressing than the protein content, the respective values for protein and non-protein fractions being 78.7 (N-deficient) and 107.4 (high-N), 32.8 and 66.6 mg N per 10 g of dry matter, respectively.

More than 70% of the estimated nitrogen of this fraction was accounted for by amides in the case of fully manured plants. In the tubers of nitrogen-deficient plants this value was 57.5%. The relative content of asparagine was increased by 18 per cent following the nitrogen manuring, that of glutamine by 43 per cent.

When the amides were excluded, arginine contributed 33 per cent

TABLE IX

Composition of the protein and the soluble non-protein fractions of potato tubers grown at two potassium levels, stored for 2 months (Exp. 1472, 1954, *var.* Noordeling)

Amino compound	Low-K potatoes						High-K potatoes					
	Protein fraction *)		Soluble non-protein fraction *)				Protein fraction *)		Soluble non-protein fraction *)			
	g am.-acid per 100 g protein (16 g N)	N as % of sum of est. am.-acid-N (incl. NH_3)	g am.-acid per 16 g am.-acid-N		N as % of sum of est. am.-acid-N		g am.-acid per 100 g protein (16 g N)	N as % of sum of est. am.-acid-N (incl. NH_3)	g am.-acid per 16 g am.-acid-N		N as % of sum of est. am.-acid-N	
			incl. the amides	excl. the amides	incl. the amides	excl. the amides			incl. the amides	excl. the amides	incl. the amides	excl. the amides
Glycine	4.8	6.8	tr	tr	tr	tr	5.8	8.0	tr	tr	tr	tr
Alanine	4.4	5.2	0.8	3.2	0.8	3.1	4.3	5.0	0.9	2.7	0.9	2.6
Serine	5.8	5.8	1.6	6.4	1.3	5.3	6.0	5.9	1.3	3.8	1.1	3.2
Cystine + cysteine	1.0	0.6	0.2	0.7	0.1	0.4	0.7	0.5	0.2	0.7	0.1	0.4
Threonine	5.1	4.5	0.6	2.6	0.5	1.9	5.8	5.1	1.1	3.3	0.8	2.4
Valine	6.8	6.2	3.1	12.8	2.3	9.6	7.6	6.7	3.1	9.3	2.3	6.9
Methionine + methionine sulphoxide	2.2	1.8	1.2	4.9	0.7	2.9	2.0	1.4	2.0	5.9	1.2	3.5
Leucines	17.5	14.2	1.6	6.5	1.1	4.4	17.9	14.1	2.7	8.0	1.8	5.4
Phenylalanine	5.5	3.5	1.2	5.0	0.7	2.7	5.3	3.3	1.7	5.0	0.9	2.7
Tyrosine	3.4	1.9	3.0	12.2	1.5	5.9	4.6	2.6	2.3	6.8	1.1	3.3
Proline	3.9	3.6	tr	tr	tr	tr	3.6	3.2	tr	tr	tr	tr
Tryptophane	1.9	1.9	—	—	—	—	1.9	1.9	—	—	—	—
Aspartic acid	8.7	6.9	2.9	11.9	1.9	7.9	8.1	6.3	3.0	9.0	2.0	5.9
Glutamic acid	9.6	6.9	5.2	21.2	3.0	12.3	10.0	7.0	11.2	33.6	6.5	19.4
Arginine	3.8	9.3	2.8	11.6	5.1	20.9	3.4	8.0	3.1	9.3	5.6	16.6
Lysine	3.7	5.3	tr	tr	tr	tr	3.9	5.5	tr	tr	tr	tr
Histidine	2.6	5.3	0.7	2.9	1.2	4.9	3.0	6.0	1.2	3.7	2.1	6.3
γ-Aminobutyric acid	0.0	0.0	5.2	21.1	4.4	18.0	0.0	0.0	8.4	25.1	7.1	21.3
Asparagine	—	—	37.7	—	50.1	—	—	—	32.3	—	42.9	—
Glutamine	—	—	21.3	—	25.5	—	—	—	19.7	—	23.7	—
Ammonia	1.6	10.1	—	—	—	—	1.6	9.4	—	—	—	—

*) 82.4% and 73.3% of the Kjeldahl nitrogen of the protein and the soluble non-protein fractions of the low-K potatoes are accounted for by the amino-acid analyses of these fractions. For the high-K potatoes these values are 84.8% and 69.7%, respectively.

of the total estimated nitrogen of this fraction in fully manured plants as against 25.9 per cent in N-deficient plants. Since the nitrogen of arginine can be easily made available in living plants, this amino-acid may function in a similar way to the amides as a temporary storage product for excess nitrogen.

TABLE X

Composition of the soluble non-protein fraction of potato tubers grown at two magnesium levels, stored for 6 months (Exp. 1220, 1951, *var.* Voran)

Amino compound	Low-Mg potatoes				High-Mg potatoes			
	Soluble non-protein fraction				Soluble non-protein fraction			
	g am.-acid per 16 g am.-acid-N *)		N as % of sum of est. am.-acid-N *)		g am.-acid per 16 g am.-acid-N *)		N as % of sum of est. am.-acid-N *)	
	incl. the amides	excl. the amides	incl. the amides	excl. the amides	incl. the amides	excl. the amides	incl. the amides	excl. the amides
Glycine	tr	tr	tr	tr	tr	tr	tr	tr
Alanine	0.6	2.1	0.6	2.0	1.1	3.2	1.1	3.1
Serine	1.1	3.4	0.9	2.9	1.3	3.9	1.1	3.2
Cystine + cysteine	tr	tr	tr	tr	tr	tr	tr	tr
Threonine	1.7	5.6	1.3	4.1	1.3	3.7	0.9	2.7
Valine	3.7	11.9	2.8	8.9	4.1	12.0	3.2	9.0
Methionine + methionine sulphoxide	tr	tr	tr	tr	tr	tr	tr	tr
Leucines	2.2	7.2	1.5	4.8	2.0	5.7	1.3	3.8
Phenylalanine	2.2	7.0	1.1	3.7	1.4	4.0	0.7	2.1
Tyrosine	3.3	10.6	1.6	5.1	4.6	13.5	2.2	6.5
Proline	1.7	5.4	1.3	4.1	1.5	4.3	1.1	3.3
Tryptophane	—		—	—	—	—	—	—
Aspartic acid	4.2	13.7	2.8	9.1	5.7	16.6	3.8	10.9
Glutamic acid	5.7	18.3	3.4	10.9	7.7	22.5	4.6	13.4
Arginine	2.5	8.2	5.1	16.4	2.4	7.0	4.8	14.1
Lysine	1.1	3.4	0.9	2.8	1.0	2.8	0.8	2.2
Histidine	1.8	5.7	3.0	9.7	1.0	2.9	1.7	4.9
γ-Aminobutyric acid	5.7	18.3	4.8	15.5	8.4	24.3	7.1	20.7
Asparagine	42.5	—	56.3	—	38.7	—	51.5	—
Glutamine	10.6	—	12.3		11.8	–	14.2	—
Ammonia	—				—		—	

*) 86.1% of the Kjeldahl nitrogen of the soluble non-protein fraction of the low-Mg potatoes is accounted for by the amino-acid analyses of this fraction. For the high-Mg potatoes this value is 94.6%.

Likewise, γ-aminobutyric acid occurred more abundantly in normal than in the nitrogen-deficient potatoes.

Although the relative contents of most other amino-acids were somewhat higher in nitrogen-deficient tubers, this was particularly true of glutamic acid.

Phosphorus. P-deficient potatoes were found to be higher in protein and particularly higher in soluble non-protein nitrogen than those with an ample P-supply (see Table IV). In agreement with the nitrogen experiment (Table V) a higher proportion of the

TABLE XI

Effect of the nitrogen nutrition of potato plants on the amino-acid content of the tubers (calculated as mg amino-acid per 10 g of dry matter, *cf* Table V)

Amino compound	Low-N potatoes		High-N potatoes	
	Protein fraction	Soluble non-protein fraction	Protein fraction	Soluble non-protein fraction
Glycine	29.0	tr	40.3	tr
Alanine	25.1	1.6	33.6	2.2
Serine	33.5	1.6	48.3	2.1
Cystine + cysteine	5.4	1.1	8.7	1.1
Threonine	32.0	2.0	43.6	2.2
Valine	33.9	4.4	43.6	5.9
Methionine + methionine sulphoxide	13.8	3.0	20.8	3.3
Leucines	79.7	5.2	109.4	7.4
Phenylalanine	24.6	—	32.9	—
Tyrosine	20.7	3.3	30.9	4.4
Proline	18.7	tr	25.5	tr
Tryptophane	8.9	—	13.4	—
Aspartic acid	51.7	9.5	70.5	14.0
Glutamic acid	48.7	32.9	65.8	35.1
Arginine	28.5	8.6	36.9	16.3
Lysine	20.7	tr	26.9	tr
Histidine	12.3	1.6	17.5	1.9
γ-Aminobutyric acid	0.0	6.7	0.0	15.6
Asparagine	—	55.3	—	141.3
Glutamine	—	14.6	—	45.4
Ammonia	8.4	—	10.7	—

nitrogen of the latter fraction occurred as amides. In the second sample no difference in relative amide content was found.

In both cases asparagine occurred much more abundantly in the soluble fraction of the P-deficient tubers than in that of tubers supplied amply with phosphorus. In one experiment glutamine contributed to the soluble nitrogen of P-deficient and normal tubers in equal amounts; in the second its contribution was lower in the case of phosphorus deficiency.

Of the amino-acids, tyrosine was found to occur in somewhat higher concentration in the soluble non-protein fraction of P-manured tubers than in that of P-deficient ones, an observation which is in agreement which earlier estimations [8]). Phenylalanine which may be considered to be the precursor of tyrosine was lower in the normal tubers.

Glutamic acid was found more abundantly in the non-protein fraction of phosphorus-deficient than in that of normal tubers. This

TABLE XII

Effect of the phosphorus nutrition of potato plants on the amino-acid content of the tubers (calculated as mg amino-acid per 10 g of dry matter, *cf* Table VII)

Amino compound	Low-P potatoes		High-P potatoes	
	Protein fraction	Soluble non-protein fraction	Protein fraction	Soluble non-protein fraction
Glycine	28.9	1.8	22.6	1.9
Alanine	21.9	2.3	16.8	1.4
Serine	31.0	2.9	23.4	2.6
Cystine + cysteine	3.2	0.7	2.7	0.5
Threonine	32.1	3.1	22.6	2.0
Valine	39.0	8.2	29.7	5.5
Methionine + methionine sulphoxide	10.2	4.5	7.8	3.8
Leucines	90.8	6.3	69.9	4.1
Phenylalanine	26.7	7.1	20.7	3.5
Tyrosine	24.0	4.3	18.0	5.2
Proline	19.2	tr	14.1	tr
Tryptophane	9.6	—	7.4	—
Aspartic acid	42.8	8.0	31.6	5.2
Glutamic acid	50.8	28.2	39.1	21.3
Arginine	17.1	12.5	13.3	5.7
Lysine	19.7	tr	15.2	tr
Histidine	16.6	3.0	11.7	2.5
γ-Aminobutyric acid	0.0	15.3	0.0	16.7
Asparagine	—	137.8	—	62.9
Glutamine	—	68.7	—	36.3
Ammonia	9.1	—	6.3	—

was true in the sample in which glutamine was lower in the P-deficient tubers. This might indicate that in potatoes the formation of glutamine from glutamic acid proceeds less readily in the case of an inadequate supply of phosphorus. In the second sample, in which no differences in glutamine content were observed, the glutamic acid content was slightly lower in phosphorus-deficient tubers.

Arginine contributed considerably more to the nitrogen of the soluble non-protein fraction in phosphorus-deficient tubers than in normal ones. Similar results have been observed in those cases in which the nitrogen supply of the plants was high. Arginine is apparently used by potato plants in a similar way to amides as a storage compound for excess nitrogen.

γ-Aminobutyric acid occurred in much lower amounts in P-deficient potatoes than in tubers from plants amply supplied with phosphorus; this was true of both samples. Since γ-aminobutyric

acid was found to be more abundant in plants amply supplied with nitrogen (see Table V) and since phosphorus-deficient tubers behaved like those amply supplied with nitrogen as far as their amide and arginine contents were concerned, it may be concluded that the formation of γ-aminobutyric acid was specifically inhibited in P-deficient potatoes.

Potassium. Potassium-deficient plants are generally considerably higher in soluble non-protein nitrogen than are those amply supplied with potassium. The protein may or may not be higher in the K-deficient plants (see Table IV).

In agreement with the nitrogen and phosphorus experiments, the amides were found to contribute more to the amino-acid-nitrogen in K-deficient tubers than in normal ones. In one case their contribution was 75.6 per cent in the K-deficient sample and 66.6 per cent in the K-manured sample; in the second sample no differences in the relative amide contents were found.

TABLE XIII

Effect of the potassium nutrition of potato plants on the amino-acid content of the tubers (calculated as mg amino-acid per 10 g of dry matter, *cf* Table VIII)

Amino compound	Low-K potatoes		High-K potatoes	
	Protein fraction	Soluble non-protein fraction	Protein fraction	Soluble non-protein fraction
Glycine	35.2	tr	31.5	tr
Alanine	39.2	3.3	35.0	2.7
Serine	43.9	7.9	35.0	6.9
Cystine + cysteine	6.7	2.6	4.1	2.4
Threonine	44.5	7.2	38.0	5.8
Valine	39.2	17.9	36.2	11.5
Methionine + methionine sulphoxide	16.6	8.9	13.4	7.4
Leucines	101.8	11.5	88.8	7.7
Phenylalanine	36.6	13.5	31.5	6.4
Tyrosine	34.6	30.9	29.2	14.6
Proline	28.6	tr	30.4	tr
Tryptophane	12.6	—	10.5	—
Aspartic acid	68.5	16.4	61.3	17.2
Glutamic acid	62.5	34.7	57.8	38.2
Arginine	27.9	27.2	25.6	23.1
Lysine	30.6	4.3	29.2	3.7
Histidine	17.3	3.5	14.0	2.8
γ-Aminobutyric acid	0.0	38.0	0.0	23.4
Asparagine	—	224.8	—	172.0
Glutamine	—	83.5	—	55.0
Ammonia	10.6	—	10.5	—

When the amides were excluded, valine was found to contribute in somewhat greater amounts to the non-protein nitrogen in K-deficient tubers than in those with an ample K-supply. Tyrosine also occurred more abundantly in the K-deficient potatoes. The latter observation is in agreement with earlier investigations in which it was found that the content of tyrosine as well as that of *o*-diphenols is considerably higher in the case of an inadequate supply of potassium.

Glutamic acid occurred in a considerably smaller concentration in the non-protein fraction of K-deficient tubers than in that of plants amply supplied with potassium.

M a g n e s i u m. Tubers from magnesium-deficient plants were also found to be higher in protein and in soluble non-protein nitrogen than those from normal plants. Asparagine contributed slightly more to the soluble non-protein nitrogen in the magnesium-deficient samples. Alanine, glutamic acid and γ-aminobutyric acid occurred less abundantly in Mg-deficient tubers, threonin, the leucines, phenylalanine, arginine and histidine occurred in somewhat greater amounts.

Effect of mineral nutrition on the nutritional value of potato tubers. From a nutritional point of view the amounts of amino-acids per unit of dry matter, contained in the protein and occurring in the free state, are important data; therefore, in Tables XI to XIV these values have been calculated per 10 g of dry matter. It will be seen that, for a number of amino-acids, the amount occurring in the protein is more than 10 times higher than that occurring in the free state. In the case of serine, cystine, threonine, valine, methionine, tyrosine, aspartic acid, glutamic acid and arginine, however, the free amino-acids contribute considerably more to the total amount of amino-acids per unit of dry matter. In the case of tyrosine, glutamic acid and arginine the free amino-acids may constitute 30–50 per cent of the total amount.

When a comparison is made between samples of varying manurial treatment, it will be seen that large differences occur. Tubers from plants amply supplied with nitrogen contained from 30 to 40 per cent higher amounts of practically every amino-acid. The content of γ-aminobutyric acid, asparagine and glutamine was more than doubled. In the case of phosphorus and potassium, the tubers from

TABLE XIV

Effect of the potassium nutrition of potato plants on the amino-acid content of the tubers (calculated as mg amino-acid per 10 g of dry matter, *cf* Table IX)

Amino compound	Low-K potatoes		High-K potatoes	
	Protein fraction	Soluble non-protein fraction	Protein fraction	Soluble non-protein fraction
Glycine	25.9	tr	23.8	tr
Alanine	23.7	3.3	17.6	1.8
Serine	31.3	6.6	24.6	2.6
Cystine + cysteine	5.4	0.7	2.9	0.5
Threonine	27.5	2.7	23.8	2.2
Valine	36.7	13.2	31.2	6.3
Methionine + methionine sulphoxide	11.9	5.0	8.2	4.0
Leucines	94.3	6.7	73.4	5.4
Phenylalanine	29.7	5.2	21.7	3.4
Tyrosine	18.3	12.6	18.9	4.6
Proline	21.0	tr	14.8	tr
Tryptophane	10.3	—	7.8	—
Aspartic acid	46.9	12.3	33.2	6.1
Glutamic acid	51.7	21.9	41.0	22.7
Arginine	20.5	12.0	13.9	6.3
Lysine	19.9	tr	16.0	tr
Histidine	14.0	3.0	12.3	2.5
γ-Aminobutyric acid	0.0	21.8	0.0	17.0
Asparagine	—	159.0	—	65.3
Glutamine	—	89.7	—	39.9
Ammonia	8.6	—	6.6	—

TABLE XV

Content of protein and soluble non-protein nitrogen of different potato varieties

Variety	N as % of dry matter		Ratio, protein-N: soluble non-protein-N
	Protein	Soluble non-protein	
Noordeling	1.02	0.85	1.20
Voran	0.89	0.97	0.92
Rode Star	0.77	0.65	1.18
Gloria	0.80	0.65	1.23
Libertas	0.68	0.54	1.26
Eigenheimer	0.84	0.76	1.11
Bintje	0.65	0.82	0.79
Bevelander	0.86	0.85	1.01
Eersteling	0.85	1.12	0.76
Industrie	0.75	0.64	1.17
Wilpo	0.80	0.69	1.16
Record	0.85	0.64	1.33

deficient plants had considerably higher contents of most amino-acids.

Amino-acid content of different potato varieties

It is a well-known fact that, independently of nutritional conditions, different potato varieties may have a widely diverging nitrogen and protein content. Table XV gives the results of an experiment with 12 potato varieties grown on the same field under similar conditions. It will be seen that not only the contents of

TABLE XVI

Composition of the soluble non-protein fractions of the potato varieties Noordeling and Voran, stored for 4 months (Exp. 1466, 1953)

Amino compound	Noordeling				Voran			
	Soluble non-protein fraction				Soluble non-protein fraction			
	g am.-acid per 16 g am.-acid-N *)		N as % of sum of est. am.-acid-N *)		g am.-acid per 16 g am.-acid-N *)		N as % of sum of est. am.-acid-N *)	
	incl. the amides	excl. the amides	incl. the amides	excl. the amides	incl. the amides	excl. the amides	incl. the amides	excl. the amides
Glycine	tr	tr	tr	tr	tr	tr	tr	tr
Alanine	0.7	2.6	0.7	2.6	1.2	3.5	1.2	3.5
Serine	0.9	3.2	0.7	2.6	1.0	2.9	0.9	2.4
Cystine + cysteine	0.3	0.9	0.1	0.5	0.2	0.7	0.1	0.4
Threonine	0.5	2.0	0.4	1.5	0.5	1.5	0.4	1.1
Valine	2.3	8.3	1.7	6.2	3.0	8.5	2.2	6.3
Methionine + methionine sulphoxide	tr	tr	tr	tr	tr	tr	tr	tr
Leucines	1.8	6.7	1.2	4.5	2.2	6.4	1.5	4.3
Phenylalanine	1.0	3.6	0.5	1.9	1.3	3.6	0.7	1.9
Tyrosine	1.9	6.8	0.9	3.3	2.5	7.1	1.2	3.4
Proline	tr	tr	tr	tr	tr	tr	tr	tr
Tryptophane	—	—	—	—	—	—	—	—
Aspartic acid	4.1	14.8	2.7	9.8	6.9	19.6	4.6	12.9
Glutamic acid	9.2	33.2	5.4	19.7	10.6	30.2	6.3	18.0
Arginine	3.3	12.0	6.7	24.2	4.1	11.8	8.3	23.7
Lysine	tr	tr	tr	tr	tr	tr	tr	tr
Histidine	1.5	5.3	2.5	8.9	1.4	4.0	2.4	6.8
γ-Aminobutyric acid	4.7	17.2	4.0	14.6	6.4	18.1	5.4	15.4
Asparagine	41.8	—	55.4	—	40.1	—	53.1	—
Glutamine	14.1	—	16.9	—	9.8	—	11.8	—
Ammonia								

*) 72.6% of the Kjeldahl nitrogen of the soluble non-protein fraction of the variety Noordeling s accounted for by the amino-acid analyses of this fraction. For the variety Voran this value s 74.5%.

TABLE XVII

Comparison between the amino-acid composition of the protein of tubers from crosses *) between different *Solanum* species × *Solanum tuberosum* and the average values for eight different samples from *Solanum tuberosum* (*var.* Noordeling)

Amino compound	Grams of amino-acid per 16 g of N					
	WT 41-7	AT	DTT 9-8	51-155-13	51-42-2	Noordeling
Glycine	3.9	3.9	3.7	4.0	3.5	5.5
Alanine	4.1	5.2	4.2	4.0	4.4	5.0
Serine	4.3	4.0	4.2	4.6	4.0	6.2
Cystine + cysteine	0.8	1.2	0.8	0.8	0.7	0.9
Threonine	4.3	4.2	4.0	3.3	3.7	6.2
Valine	7.9	7.8	8.9	8.4	8.3	6.7
Methionine + methionine sulphoxide	1.8	1.9	1.8	1.8	1.7	2.4
Leucines	18.2	17.4	20.2	18.8	19.1	16.5
Phenylalanine	—	—	—	—	—	5.2
Tyrosine	4.6	5.7	—	5.8	6.1	4.5
Proline	5.1	4.0	4.2	5.5	4.0	4.0
Tryptophane	—	—	—	—	—	1.9
Aspartic acid	10.7	12.0	11.5	11.5	11.6	9.5
Glutamic acid	10.3	10.7	10.6	8.8	8.6	9.7
Arginine	3.9	3.6	3.8	3.6	4.0	4.3
Lysine	3.4	3.3	3.9	3.7	3.2	4.2
Histidine	—	—	—	—	—	2.7
γ-Aminobutyric acid	0.0	0.0	0.0	0.0	0.0	0.0
Asparagine	—	—	—	—	—	—
Glutamine	—	—	—	—	—	—
Ammonia	1.5	1.5	1.6	1.4	1.5	1.6

*) Samples received from Dr. H. J. Toxopeus, Institute of Agricultural Plant Breeding Wageningen. WT 41—7 = *S. andigenum* × *S. tuberosum;* AT = *S. andigenum* (resistant to the potato root eelworm) × *S. tuberosum;* DTT—9—8 = (*S. demissum* × *S. tuberosum*) × *S. tuberosum*; 51—41—2 = (*S. andigenum* × *S. demissum*) × *S. tuberosum*; 51—153—13 = (*S. demissum* × *S. rybinii*) repeatedly backcrossed with *S. tuberosum*. All varieties of *S. andigenum* used are very different from each other.

protein and soluble non-protein nitrogen vary considerably but also the ratio, protein nitrogen: soluble non-protein nitrogen. The latter is of considerable interest from a nutritional point of view as the soluble non-protein fraction consists of more than 50 per cent of amides which have a low nutritional value. The variety Noordeling which was employed in most of the above-mentioned experiments had a ratio, protein nitrogen : soluble non-protein nitrogen, of 1.20 as compared with 0.92 in Voran.

In a subsequent experiment, in which tubers of the variety Noordeling contained 102.7 mg protein-N and 69.7 mg soluble non-protein-N and those of the variety Voran 54.7 mg protein-N and

54.6 mg soluble non-protein-N per 10 g of dry matter, the non-protein fraction was analysed for amino-acids. Table XVI contains the results of this investigation. It will be seen that the difference between the two varieties in amino-acid composition of this fraction was relatively small. Asparagine and particularly glutamine contributed considerably more to the nitrogen of this fraction in the Noordeling than in the Voran varieties. This is in agreement with the nutrition experiments described above in which a high concentration of soluble non-protein nitrogen was correlated with a high proportion of the nitrogen of this fraction occurring in the amide form.

No data concerning the protein content of the two varieties are available. Protein analyses were made on a number of crosses between different *Solanum* species × *Solanum tuberosum*. For comparison the average values for 8 analyses of the variety Noordeling are given (see Table XVII). It will be seen that small differences occur in the contents of glycine, serine, threonine, valine, the leucines and aspartic acid.

DISCUSSION

The purpose of the present study was to investigate the effect of the mineral nutrition of potato plants on the amino-acid composition of the protein and the soluble non-protein fractions of the tubers. Such information is not only important from a plant-physiological point of view, but is also of interest in the study of human and animal nutrition. In many countries the potato is one of the main foodstuffs and according to Groot [3]) 10 25% of the total amount of protein consumed in human nutrition in the Netherlands consists of potatoes.

The separation between the protein and the soluble non-protein fractions was made by heat coagulation after the addition of a few drops of acetic acid and a small amount of sodium chloride. The amount of precipitated nitrogen compound was found to agree well with the amount precipitated by trichloroacetic acid. The latter is generally considered to represent the protein fraction.

The protein as used in the present investigation was of a satisfactory purity (approximately 90%). In contrast to the alcohol-insoluble fraction, analysed by Thompson and Steward, [11]) it did not contain the peptides.

From the investigations of Kiesel *et al.*[7]), Groot *et al.*[4]), and Hofstee[5]) it has become clear that the protein of the potato tuber consists of at least two different fractions, viz. one with globulin characters (insoluble in water after dialysis), the so-called tuberin, and a second which behaves like an albumin (soluble in water after dialysis), the so-called tuberinin. These fractions occur in a ratio of 7 : 3; both are coagulated by heat so that it may be concluded that the protein analysed in the present investigation consists of a mixture of tuberin and tuberinin. Kiesel *et al.* have analysed both fractions for a number of amino acids; they found no differences between them.

Although, in the past, a number of analyses have been made of the amino-acid composition of potato protein, it was not before the development of the quantitative paper-chromatographic method by Thompson and Steward[12]) [13]) that the complete composition of potato protein was given[14]). It must be stressed, however, that these authors did not hydrolyse the isolated protein

TABLE XVIII

Comparison of the amino-acid composition of potato-tuber protein as obtained by various authors (g amino-acid per 16 g protein-N)

Amino-acid	Sjollema and Rinkes[9]) (tuberin)	Kiesel *et al.*[7])		Groot[3])	Slack[10])	Thompson and Steward[14])	Present paper (average of 8 samples)
		tuberin	tuberinin				
Glycine	—	—	—	—	—	5.6	5.5 ± 0.16
Alanine	4.9	—	—	—	—	6.2	5.0 ± 0.29
Serine	—	—	—	—	—	3.7	6.2 ± 0.30
Cystine + cysteine	4.4	—	—	0.9	2.1	—	0.9 ± 0.11
Threonine	—	—	—	6.9	5.9	3.2	6.2 ± 0.21
Valine	1.1	—	—	7.6	6.1	7.7	6.7 ± 0.18
Methionine + meth. sulphoxide	—	—	—	2.6	2.3	1.5	2.4 ± 0.19
Leucines	12.2	—	—	14.6	17.5	17.6	16.5 ± 0.39
Phenylalanine	3.9	—	—	5.9	6.6	12.7	5.2 ± 0.10
Tyrosine	4.3	3.6	3.7	—	—	3.9	4.5 ± 0.22
Proline	3.0	3.9	4.2	—	—	3.5	4.0 ± 0.21
Tryptophane	—	—	—	2.3	1.6	—	1.9 ± 0.04
Aspartic acid	—	—	—	—	—	12.0	9.5 ± 0.45
Glutamic acid	4.6	—	—	—	—	14.6	9.7 ± 0.09
Arginine	4.2	6.6	6.4	4.8	6.0	6.0	4.3 ± 0.37
Lysine	3.3	—	—	3.6	7.7	8.0	4.2 ± 0.18
Histidine	2.3	2.0	2.2	2.2	2.2	—	2.7 ± 0.11
Ammonia	1.8	—	—	—	—	—	1.6 ± 0.03

but the residue which was left after thorough extraction of ground potato tissue with hot alcohol. This treatment presumably left the peptides in the insoluble fraction. A comparison of the results obtained by various authors is given in Table XVIII.

It will be seen that, for a number of amino-acids, the values found by Thompson and Steward [14]) agree satisfactorily with those found in the present investigation. For alanine, phenylalanine, aspartic acid, glutamic acid, arginine and lysine, higher values were found by Thompson and Steward [14]); for serine, threonine, and methionine, lower values were found by these authors. In the case of threonine, methionine, and phenylalanine the values obtained in the present study are in agreement with those of the other authors mentioned The value for phenylalanine of Thompson and Steward [14]) which was determined chromatographically is presumably too high, due to contamination with some leucine which produces, weight for weight, much more colour than phenylalanine. Phenylalanine should be estimated by a separate method. The values for arginine and lysine which are considerably lower in the present study then in the work of Thompson and Steward [14]) are similar to those of Groot [3]) and Sjollema and Rinkes [9]), while the much higher values of Thompson and Steward [14]) agree with those of Slack [10]).

It must be stressed that Groot [3]) analysed the same variety as used in the present investigation, viz. Noordeling. Although it is possible that the protein of different varieties of potato and that of potatoes grown under widely differing climatic conditions is different in amino-acid composition, the evidence obtained so far for this hypothesis is very poor. Steward and Thompson [11]) give the values for two varieties to show that varietal differences in amino-acid composition of tuber protein occur. It is highly probable, however, that the differences recorded by these authors depend on an erroneous calculation. In the protein of their variety Sebago 14 per cent amide-N was found, in the case of the variety Katadin no value for amide-N is given. On the assumption that the amide content of the latter variety would also have amounted to 14 per cent, all values for this variety have to be multiplied by 0.86 to make them comparable to those for the variety Sebago. When this calculation is carried out almost exactly the same amino-

acid composition of the tuber protein is found in both varieties.

The data of Table XVII of the present paper give some indication that the amino-acid composition of the protein from crosses between different *Solanum* species × *Solanum tuberosum* is slightly different from the average value found in Noordeling. The differences are small, however, and since the tubers of the crosses and those of Noordeling were grown in different years, and on different sites, it is highly questionable whether these data may be used as evidence for varietal differences in the protein composition of potato tubers.

The main conclusion of the present investigation is that the amino-acid composition of potato protein is independent of the mineral nutrition of the plants. This was found to be true of nitrogen, phosphorus and potassium supply. Although the protein content of tubers from plants with pronounced symptoms of N-, P-, or K-deficiency may be quite different from that of plants with an ample supply of these nutrient elements, the amino-acid composition of the protein of these tubers is not affected by the mineral nutrition.

The composition of the soluble non-protein fraction is much less constant than that of the protein fraction. It is affected by the mineral nutrition, variety, and presumably by other factors like climatic conditions, age, *etc.*

Tubers from plants with a liberal nitrogen supply, which generally have a considerably higher content of soluble non-protein nitrogen, were found to contain a greater proportion of this fraction in the form of amides than those from plants with a moderate or a poor nitrogen supply (70.8 and 57.5% respectively in the samples tested); the glutamine content was increased even more than that of asparagine. Apparently both amides may be used as storage products for nitrogen. This conclusion is not in agreement with previous statements in the literature according to which asparagine should have the main function in the storage of ammonia.

In the case of phosphorus deficiency and potassium deficiency the potato tubers were higher in protein and particularly in soluble non-protein nitrogen. The contribution of the amides to the latter fraction was considerably greater in phosphorus- and potassium-deficient tubers than in those with a normal supply of these nutrients. This rise in relative amide-N was completely accounted for by asparagine in the case of P-deficiency. In one experiment

the relative glutamine content was even considerably lower in P-deficient tubers. In the case of K-deficiency both asparagine and glutamine contributed to the rise in relative amide content.

Of the free amino-acids, the relative contents of tyrosine, glutamic acid, arginine and γ-aminobutyric acid were found to be affected by the mineral nutrition.

Tyrosine occurred much more abundantly in K-deficient potatoes than in those with a normal K-supply. This is in agreement with earlier investigations [8]) in which the high tyrosine content of K-deficient potatoes was found to be related to the high liability of such tubers to enzymatic blackening. In phosphorus-deficient tubers the tyrosine content was lower than in normal tubers.

Glutamic acid occurred more abundantly in the non-protein fraction of nitrogen-deficient potatoes than in that of normal tubers. Potassium-deficient potatoes which are always rich in non-protein nitrogen behaved in a similar way: their relative glutamine content was considerably lower than that of tubers with an ample K-supply. Phosphorus-deficient tubers, however, although also considerably higher in soluble non-protein nitrogen than tubers with a normal P-supply were as high as or higher in relative glutamic-acid content than the latter.

Arginine was found in greater amount in the non-protein fraction of potatoes with an ample nitrogen supply than in nitrogen-deficient tubers; P-deficient and K-deficient potatoes behaved in a similar way. This amino acid, like the amides, is apparently associated with ammonium storage.

γ-Aminobutyric acid contributed considerably more to the soluble non-protein nitrogen in tubers from plants dressed amply with nitrogen than in nitrogen-deficient tubers. This was true to some extent also in the K-deficient potatoes as compared with the K-manured ones, but the phosphorus-deficient tubers behaved quite differently; their relative γ-aminobutyric acid content was quite low when compared with normal plants.

From a nutritional point of view it is important to know that the free amino-acids in some cases may contribute considerably to the total amount of amino-acids contained in the potato. In the case of amino-acids essential to human nutrition (threonine, valine, methionine, leucine and iso-leucine, phenylalanine, tryptophane and lysine) this is true of valine, methionine, phenylalanine and

possibly tryptophane. Lysine, which, from a nutritional point of view, may occur in somewhat too low a concentration in potato protein, is only present in trace amounts in the soluble non-protein fraction. The fact that mineral nutrition may have a pronounced effect on the protein content, and as a result of this on the amino-acid content of potatoes is also important for the human and animal nutrition specialist. Tubers from plants supplied amply with nitrogen may contain 30 per cent more of the essential amino-acids than those from N-deficient plants. Tubers from P-deficient and K-deficient plants behave in general like those from plants manured with ample nitrogen. It must be stressed that this effect of mineral nutrition on amino-acid content works mainly through the protein content of the tuber and only to a minor extent through the soluble non-protein fraction. The latter fraction consists of 50–60 per cent of amides which have only a minor value in human nutrition; increased nitrogen supply may raise this percentage to 70–75 per cent.

SUMMARY

The paper-chromatographic method of Thompson and Steward [12] [13] has been used for the estimation of the amino-acid composition of the protein and of the free amino-acids in potato tubers grown under carefully controlled manurial conditions. A comparison has been made between tubers from plants deficient in nitrogen, phosphorus and potassium, respectively, and those from plants grown on the same field with an optimum supply of these elements.

Although the mineral nutrition of the potato plant may have a pronounced effect on the protein content of the tubers, the amino-acid composition of the protein was found to be independent of the supply of nitrogen, phosphorus and potassium.

The amino-acid composition of the soluble non-protein fraction was found to vary considerably under the influence of a different mineral nutrition. Conditions which increase the nitrogen content of the potato (ample N, deficiency in P or K) increase the proportion of the amides in the soluble non-protein fraction. Both asparagine and glutamine were found to be associated with nitrogen storage.

Arginine and in some cases, γ-aminobutyric acid, behaved like the amides; in P-deficient tubers, however, the relative content of γ-aminobutyric acid was much lower than in normal tubers, although the total non-protein-N was high.

The tyrosine content of P-deficient tubers was lower, that of K-deficient tubers much higher than normal. Glutamic acid contributed much less

abundantly to the soluble non-protein fraction when the nitrogen supply of the plants was high.

From the point of view of human and animal nutrition it is important to know that in some cases the free amino-acids may contribute considerably to the total amino-acids of the potato tubers, furthermore, that the mineral nutrition may have a pronounced effect on the amino-acid content of the potato, mainly through its effect on the protein content.

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THE RELATIONSHIP BETWEEN THE GROWTH AND MINERAL COMPOSITION OF THE FOLIAGE OF JAPANESE LARCH (*LARIX LEPTOLEPIS*, MURR.)

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INTRODUCTION

The relationship between mineral composition and growth in plants has received considerable attention as a basis for the diagnosis of mineral nutrient deficiencies (cf Goodall and Gregory [2]), Lundegårdh [5])). When only one nutrient is limiting growth and the remainder are supplied in optimum amounts, the response in growth and in the concentration of the deficient nutrient in the tissues (foliage) to increasing supplies of that nutrient usually follows a curve of diminishing returns (for exceptions cf Steenbjerg [9]) [10])). Under exactly comparable conditions of sampling, the concentration of the deficient nutrient therefore provides a measure of the extent of the deficiency (exceptions as above). In the field, however, such conditions rarely apply; more often than not, more than one nutrient is limiting, in the sense that positive responses in growth can follow the separate additions of different nutrients and furthermore, there are often appreciable interactions between nutrients in their effect on both growth and their concentration in the foliage. In such cases it has been claimed that interpretations based on the concentration of a single nutrient may lead to serious errors in diagnosis. According to Lundegårdh [4]) for example, the increment in yield following a given fertilizer application to plants with a particular concentration of that nutrient is markedly affected by the level of other nutrients. Other investigators however, (Macy [8]), Ulrich [14])) claim

that for each nutrient there is a particular (so-called critical) concentration, more or less *independent of the level of other growth factors*, above which luxury consumption occurs and below which growth increases as the supply of that nutrient is increased. Still others maintain that where variations in more than one nutrient are concerned, the question of balance between nutrients must also be taken into account (cf Thomas [12])). Despite these differences in interpretation, however, inspection of the experimental data published by many investigators reveals a common feature which offers an alternative approach to the diagnosis of mineral nutrient deficiencies. Over much of the deficient range of a nutrient there is a linear relationship between the concentration of that nutrient and growth, or at least the correlation between these quantities reveals a significant linear component of the regression of one on the other. When more than one nutrient is deficient but attention is directed only to one (usually the most deficient or limiting factor), significant linear correlations are still obtained between the concentration of that nutrient and growth, though the slope of the regression line and the range over which it applies may vary according to the level of the other growth factors. This is clearly shown, for example, in the data of Mitchell and Chandler [6]) for various hardwood species growing on sites differing in fertility, treated with graded dosages of a nitrogenous fertilizer. For each species a definite relationship was established (diminishing returns) between growth increment and nitrogen concentration in the foliage but the range of concentration over which this relationship was effectively linear and the slope of the line varied from site to site. A somewhat similar result was obtained by Leyton [3]) for Sitka spruce growing on a nitrogen-deficient site: over the range studied, the nitrogen concentration in the needles was significantly correlated with needle dry weight but the line relating these two quantities was displaced according to whether phosphate had been applied to the trees, or not.

It would appear from these and many other examples (cf *e.g.* Chapman [1])), that the presence of a significant positive correlation between growth and the concentration of a particular nutrient may be taken as reasonable evidence for a deficiency in that nutrient more or less independent of the level of other nutrients within the range concerned. The concentration alone however will

not provide a direct measure of the degree of response when the supply of the nutrient is increased though it is obvious that, within the range covered by the correlation, a lower concentration means a greater deficiency in that nutrient.

It may be argued further that specially designed fertilizer trials are not always necessary to establish the nature of mineral deficiencies if a suitable range of material already exists covering the required variation in growth and concentration. Thus, taking advantage of existing variations in the growth of Sitka spruce in an even aged plantation on a presumably heterogeneous site, Leyton[3]) found a significant positive correlation between height growth and nitrogen concentration in the needles; that this indicated a nitrogen deficiency was supported by a marked response in growth when the nitrogen status of the trees was improved either by fertilizing or by other ameliorative treatments, in which cases the linear correlations were no longer significant.

Unfortunately, in the majority of investigations reported so far, the relation between growth and nutrient concentration (as distinct from growth and supply) has been restricted to a single, usually the so called limiting, nutrient and as far as is known, few attempts have been made to represent algebraically by means of regression equations, relationships involving two or more nutrients. As will be seen, the present investigation with Japanese larch provides an excellent opportunity for analysing such relationships.

EXPERIMENTAL MATERIAL AND METHODS

Trees were selected from a pure plantation established in 1948 from 2 year old transplants on Hackness (Broxa) Moor in North East Yorkshire. The soil is a heathland podzol overlying Lower Calcareous grit (Jurassic) and is very deficient in bases and in available nitrogen (for details of this area cf Leyton[3]). Before planting the area was completely ploughed and at the time of planting received a dressing of 1½ ounces of ground mineral phosphate per tree; 3 to 4 years later, part of the plantation received a further dressing of nitro chalk (3 4 ozs. per tree) and in addition, a small area was treated with ground limestone. As a result of this varied treatment, combined probably with a certain amount of hetero-

geneity in soil and in planting stock, the growth of the trees has been extremely variable.

About the middle of August 1954, when the trees had more or less completed their extension growth for that year, 26 trees were selected, ranging in height from 65 to 351 cm. From each tree one lateral shoot was taken from the upper most whorl immediately below the terminal shoot and after drying in an oven, the needles were ground in a mill and analysed for nitrogen, ash, phosphorus, potassium and calcium.

EXPERIMENTAL RESULTS AND STATISTICAL ANALYSIS

In Figure 1, the concentrations of the various nutrients in the needles (% dry weight) have been plotted against the corresponding tree heights. Inspection of this figure suggests that in all cases, with the possible exception of calcium, height growth and nutrient concentration are linearly related; these relationships are confirmed by the following simple correlations: —

	Simple correlation coefficients *)					
	%N	%K	%P	%Ca	%Ash	% Ash residue **)
Tree height. .	.818	.792	.774	.441	.859	.962

*) At 1% level of significance $r = .487$ for 25 degrees of freedom.

**) Since the ash already contains the K, P and Ca factors, an attempt has been made to evaluate the content of other ash constituents by subtracting the equivalents K_2O, P_2O_5 and CaO from the ash contents: in the absence of precise information as to the way in which these elements are combined in the ash, the resulting 'ash residue' is, of course, an approximate value only.

Calculation of the multiple regression of tree height on the concentration of the above constituents in the needle dry weight (omitting the ash) yields the following equation: —

$$\hat{Y} = 96.34\,X_1 + 95.51\,X_2 + 371.02\,X_3 - 62.06\,X_4 + 63.39\,X_5 - 170.04$$

where $\hat{Y}$ = estimated tree height (cm)
X_1 = N % D.Wt.
X_2 = K % D.Wt.
X_3 = P % D.Wt.
X_4 = Ca % D.Wt.
X_5 = "Ash residue" % D.Wt.

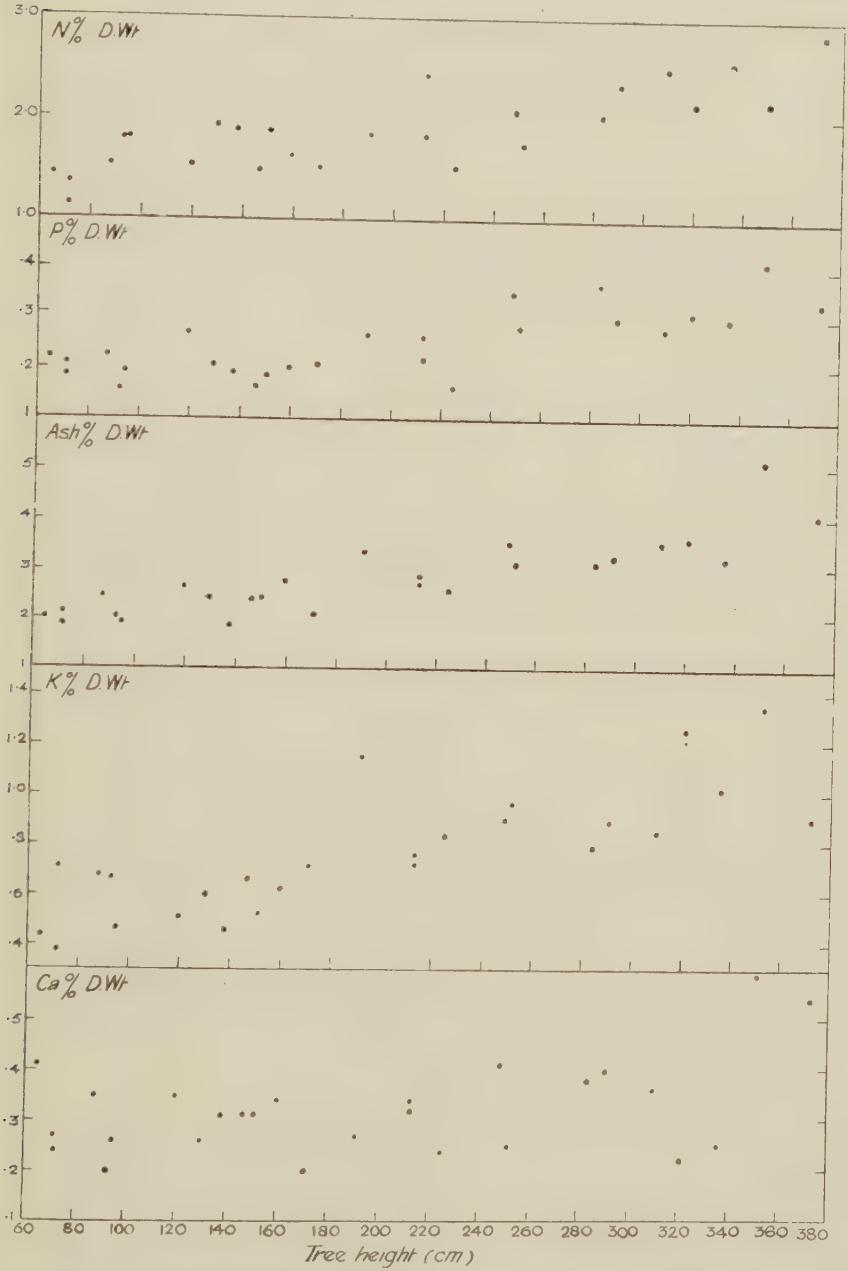

Fig. 1. The relationship between growth and mineral concentration in the foliage of Japanese larch.

The standard error of the estimate is $\pm$ 38.07 cm and the coefficient of the multiple correlation (between actual and estimated heights) is 0.934, *i.e.* highly significant. This equation alone however provides no information as to the significance of the contribution made by the various mineral constituents in the assessment of height. Such information is obtained by inspection of the partial correlation coefficients between height and each factor, independent of the remaining factors or, better still, by testing the effect of successively eliminating each of the factors on the precision of the estimate.

Analysis of the data reveals that the concentrations of the above nutrients in the larch needles are not themselves independent; there are for example, significant simple correlations between the concentration of nitrogen and that of phosphorus ($r = .602$), between nitrogen and the ash residue ($r = .704$), between potassium and ash residue ($r = .711$) and between phosphorus and calcium ($r = .640$). Calculation of the partial correlation coefficients yields the following values (A = ash residue): —

Between height and	Independent of	Partial correlation coefficient
% N	K, P, Ca, A	.657
% K	N, P, Ca, A	.356
% P	N, K, Ca, A	.341
% Ca	N, P, K, A	—.113
% A	N, P, K, Ca	.301

At 1% level of significance, $r = .537$ and at 5% level of significance, $r = .423$ for 20 degrees of freedom.

It would appear from these results that, as far as significant partial correlations are concerned, only nitrogen merits attention as the factor linearly related to tree height independent of the other factors. However, as will be seen later, nitrogen alone does not provide the best estimate of height.

Tests on the precision of the estimate following the successive elimination of particular factors are based on the fact that the sum of squares representing deviations from the regression function (*i.e.* error) is proportional to $(1 - R^2)$, where R is the multiple correlation coefficient. The effect of eliminating a particular factor is to increase this sum of squares and the significance of the resulting loss in precision can be estimated by applying the F test to the mean

squares of 1) the difference between the sums of squares due to regression (= the difference between the two values for $1 - R^2$), with 1 degree of freedom, and 2) the original error or deviation from the regression. Representative calculations are illustrated in Table I below: —

TABLE I

Loss in precision of height estimates following elimination of particular factors

Factors	R	$1-R^2$	d.f.	Error mean square	Difference $1-R^2$	Difference mean square	F
N, K, P, Ca, A	.934260	.127156	20	.0063578	.012637	.012637	.199
N, K, P, Ca	.927473	.139793	21	.0066568	.000002	.000002	.003
N, K, P	.927472	.139795	22	.0063543	.021505	.021505	3.384
N, K	.915810	.161300	23	.0070130	.169249	.169249	24.134
N	.818160	.330549	24	.0137728			

According to the data presented in Table I, and similar calculations, none of the contributions made by the P, Ca or ash residue factors to the regression prove to be significant and height can therefore be represented adequately in terms of the nitrogen and potassium concentrations only. The fact that potassium yields no significant partial correlation with tree height is probably bound up with some interrelation with the ash residue factor (*e.g.* incomplete removal of the K-content as K_2O). When the latter is excluded, the partial correlation between tree height and potassium concentration, independent of N, P, and Ca, is now significant (r .533); that for nitrogen is also significant (r .702) whilst those for phosphorus and calcium are well below the significant level ($r = .293$ and $-.0035$ resp.).

Calculation of the new regression, including only the N and K factors yields the following equation: —

$$\hat{Y} = 123.27\,X_1 + 188.69\,X_2 - 180.91$$

where, as before, $\hat{Y}$ = estimated tree height (cm)
X_1 = N % D.Wt.
X_2 = K % D.Wt.

The standard error of the estimate is ± 40.9 cm and the multiple correlation coefficient is .916. No further improvement in the accuracy of the estimate has been obtained by including various

combinations of the nutrient factors *e.g.* N^2, K^2 or the product $N \times K$, though a similar degree of precision is obtained ($R = .917$) when potassium is replaced by the ash content (since the contents of potassium and of ash in the needles are significantly related to each other, it is very probable that the ash contributes to the regression almost wholly through its potassium content). The partial correlations between height and nitrogen concentration, independent of potassium and between height and potassium concentration, independent of nitrogen are both highly significant ($r = .754$ and .716 respectively).

DISCUSSION

According to the statistical calculations presented above, the height of the larch trees can be significantly related to the concentrations of nitrogen and potassium in the needles by the formula:

$$\text{Height (cm)} = 123.27\,\%\,N + 188.69\,\%\,K - 180.91$$

The error, amounting to approximately 16% of the total variance of height, must include unknown experimental errors in analysis besides the influence of other growth factors.

In the interpretation of this regression equation note must be taken of the basic assumption that height is dependent on the concentration of these nutrients in the needles. However, in view of the many previous investigations into the nutrient relations of plants and the arguments presented in the introduction, it is very probable that the nutrient supply to the trees (or, more strictly, the nutrient uptake by the trees) governs both height growth and nutrient concentration in the needles and that these are therefore related. Thus, under the given experimental conditions, any increase in the concentration of either nitrogen or potassium in the needles would be accompanied by an increased height growth, *independent of the concentrations of the other nutrients*. It might be argued further that such increased concentrations could be brought about by applying the appropriate fertilizers to the trees; although at present no direct evidence is available to substantiate this point, analogy with previous experiments suggests that such a response is very probable. Furthermore, whilst little attention has been paid to the relation between tree growth and the various fertilizers applied to this plantation (the nature of the trials did not allow for

a strict analysis of the response) there is no doubt that tree growth is best on the area treated with nitrochalk.

The above arguments suggest that the trees in this plantation are suffering from a deficiency in both nitrogen and potassium, especially in the former, but the degree of response to particular applications of these nutrients will of course depend upon the relation between supply and concentration and also on the influence of one nutrient on the concentration of the other. Within the range covered by a significant linear correlation between growth and nutrient concentration, supply and nutrient concentration also tend to be linearly related (cf *e.g.* the data of Mitchell[7]); in this respect therefore the claims of Chapman[1]) as to the applicability of the law of limiting factors would appear to be confirmed, at least when each nutrient is considered independently. According to these arguments, an increase in the supply of either nutrient would produce an increase in height and in the absence of significant contribution to the regression made by the factors N^2, K^2 or the product $N \times K$, it would appear that as far as their *concentrations* are concerned, the effect of both nutrients together is additive. On the other hand it is not possible to predict by analysis of the above data, how much a particular application of say, nitrogen, would influence the concentration of potassium in the needles or vice versa: this could only be determined by actual fertilizer trials. It must also be emphasized that the above deductions can only be applied under the given experimental conditions and within the range of concentrations and balance of the various nutrients concerned. The concentration ranges concerned (% dry weight) are as follows: – nitrogen (1.13 to 2.88), phosphorus (0.157 to 0.417), potassium (0.38 to 1.35) and calcium 0.20 to 0.59.

It would be interesting to compare these findings with the results of other investigations into the nutrient relations of Japanese larch but unfortunately the few which have been reported refer mainly to manurial trials with young seedlings on soils of different fertility (Uchida *et al.*[13]), Van Goor[15]) [16])). For certain Dutch heaths, Van Goor[17]) reports a positive relation between tree growth and total soil phosphate content but suggests that little response to phosphate is to be expected when the total content exceeds 50–60 mg P per 100 g; it is of interest to note that the soil concerned in the present investigation contains approxi-

mately 50–70 mg P per 100 g and that the trees were also given a phosphate dressing at the time of establishment so that the absence of evidence for a phosphate deficiency may not be unexpected. Van Goor [15]) provides data on the nutrient composition of seedlings grown in pots with different levels of nitrogen and phosphorus from which he concludes that the N/P balance is of considerable importance in growth but no evidence for this has been found in the present investigation; because of differences in age of the material, little would be gained by a further comparison of the results. Suchting *et al.* [11]) investigated among other things the mineral composition of the needles of more comparably aged trees (6 years old) sampled at different times during the growing season but unfortunately deal only with unfertilized and completely fertilized material, and the data therefore, provide little information as to the relation between concentration and response in respect of particular nutrients.

As far as nitrogen is concerned there is little doubt that in heathland soils of the kind concerned in the present investigation the availability of this nutrient is very low (Leyton [3])) and the occurrence of a nitrogen deficiency limiting the growth of Japanese larch is not unexpected especially when one takes into account the better growth of the trees on the area treated with nitrochalk. The concept of a potassium deficiency on these soils however is relatively new but so far as is known no field trials on this species with this fertilizer have been carried out.

It has been the aim of this investigation to establish and interpret a regression analysis based on existing material without particular reference to the fertilizer response side of the problem. Obviously the arguments presented in this way cannot be substantiated without the evidence from field trials with the appropriate fertilizers. Such trials are in hand*).

SUMMARY

Investigations on young Japanese larch trees growing on a heterogeneous site, have revealed significant linear correlations between the height of the trees and concentrations of nitrogen, phosphorus, potassium and ash in the needles, but only doubtfully so in the case of calcium. Analysis of the multiple

*) At the time of going to press, results from a current factorial experiment with N- and K-fertilizers definitely indicate a response in growth to both nutrients, but a full statistical analysis is not yet available.

regression of height on the concentrations of these nutrients suggests that only the contributions made by nitrogen and potassium are significant and a regression equation relating height to the concentration of these two nutrients in the needles has been calculated. The function $123.27\% N + 188.69\% K - 180.91$ provides an estimate of tree height with a standard error of $\pm$ 40.9 cm and with a highly significant multiple correlation coefficient of .916. It is argued from these results that, under the conditions of the experiment and the range of concentrations encountered, the height growth of the trees is limited by deficiencies in both nitrogen and potassium.

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PLANT ROOT EXCRETIONS IN RELATION TO THE RHIZOSPHERE EFFECT

I. THE NATURE OF ROOT EXUDATE FROM OATS AND PEAS

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INTRODUCTION

It is generally accepted that the main factors for the stimulation of micro-organisms in the rhizosphere are the excretion of organic substances and the sloughing off of root hairs and epidermal cells. Investigations on the nature of root exudates show that a wide range of substances are excreted.

Among the early reports of root excretions was that of Roberts [22]) who demonstrated that root hairs were capable of bursting to eject part of their contents and then, by the cell walls springing back into place, resuming their normal appearance. Such a finding, if true, reveals the difficulty of separating true exudates through cell walls from the material released by autolysis of epidermal cells. Lyon and Wilson [15]) found that roots in water cultures excreted relatively high amount of organic matter including organic nitrogen, while Cranner [6]) reported that phosphatides were excreted by germinating seeds and by the roots of both seedlings and mature plants.

Virtanen and Laine [30]) demonstrated that nodulated legumes excreted nitrogenous substances, including aspartic acid, which could be utilized by non-legumes. Wilson [34]) in reviewing the work of Virtanen and others on nitrogen excretion by legumes concluded that this excretion occurs only under very

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special conditions of light, temperature and length of day. Tamm and Schendel[27]) reported the excretion of amino acids by nodulated legumes 7 to 12 weeks after planting. However, these must be regarded as special cases resulting from the symbiosis between *Rhizobium* and the host and not as true root excretions.

Loehwing[13]) in his review on root interactions states that many early reports on toxic secretions by roots were unsound and the injurious effects were due to factors such as nitrogen unavailabilty and detrimental effects on soil structure. However, Osvald[20]) reported that the roots of *Agropyron repens* excreted toxic substances inhibiting the germination of winter rape, *Taraxacum* and oats. Another report came from Roux[24]) who demmonstrated that the roots of *Trachypogon plumosus* excreted into sand unidentified toxic substances which inhibited germination and adversely affected the growth of another plant, *Tagetes minuta*. Evidence of excretions toxic to soil micro-organisms was found by Timonin[28]) who found that hydrocyanic acid was released by the roots of flax varieties resistant to *Fusarium* and *Helminthosporium*.

Reports of non-toxic excretions from living roots are numerous, indicating the importance of these substances in influencing the micro-organisms around the roots. West[33]) found that flax seedlings grown in sterile nutrient solution excreted significant quantities of thiamin and biotin, explaining the presence in the rhizosphere of organisms which require these vitamins. Rogers, Pearson and Pierre[23]) in a study of the absorption of organic phosphate by corn and tomato roots demonstrated that a considerable number of intact cells are shed by the root cap and these cells liberate an exoenzyme capable of releasing phosphate from glycero-phosphate and nucleic acid. Material of this kind, although an exudate from sloughed-off cells, is difficult to separate from true root excretions and is best considered as such.

The excretion by potato roots but not by beet, rape, lupin, mustard nor oats, of a substance stimulating the hatching of potato eelworm cysts was demonstrated by O'Brien and Prentice[19]). Calum *et al.*[5]), during an investigation on the chemical nature of this factor, found it excreted by the roots of tomato and *Solanum nigrum*.

Working with excised root tips, Lundegårdh and Sten-

lid [14]) showed that glucose, flavanones and nucleotides were excreted by wheat while peas excreted glucose and nucleotides but not flavanones. The authors suggest that the differences in constitution of the exudates of the roots of peas and wheat may partly account for their different rhizosphere populations. This work, however, on the exudates from excised roots suspended in distilled water need not necessarily represent the normal behaviour of the roots, particularly as Achromieko [1]) demonstrated that the behaviour of even intact roots in water culture was not always as found in soil.

Nutman [18]) reported that red clover, lucerne and vetch either nodulated or supplied with nitrate, excreted substances giving a colour reaction with bentonite. He suggested that this colour could be due to the secretion of amino compounds or carotenoids. Apart from flax, non-legumes gave no colour reaction. The excretion by germinating pea seeds of lysine, arginine, methionine, uridine (or cytidine or both), adenine and guanine (or the corresponding nucleosides) was clearly demonstrated by Fries and Forsman [9]). They showed that these substances were exuded by the root tip as well as the cotyledons. The nucleic acid derivatives appeared to be transferred from the cotyledons to the roots and it is suggested that as these plants develop, the nucleic acid supply from the cotyledons becomes exhausted and the nature of the exudate will alter, causing a change in the rhizosphere flora.

Amino-acid excretion by excised roots of maize growing aseptically has been reported by Kandler [10]), and by pea root tips suspended in distilled water by Forsman [7]). The latter has also found amino-acid excretion by peas growing in water culture. Katznelson, Rouatt and Payne [11]) found that dessication and subsequent rewetting of the sand in which tomatoes, soy bean, barley or oats were growing resulted in the excretion of the amino acids, glutamic, aspartic, leucine, alanine, cysteine, glycine, lysine, phenylalanine and proline and a reducing compound of R_f value identical with glucose. As this work was not carried out under aseptic conditions, it is possible that the rhizosphere population could play a part in this liberation. However, the authors mention that small amounts of ninhydrin-reactive material was produced by roots growing aseptically which indicates that the compounds released upon dessication are probably the result of direct excretion.

In a study of the growth factor requirements of mycorrhizal fungi of forest trees, Melin [16]) demonstrated that cultures of excised pine and tomato roots produced an unidentified substance, factor "M", which promoted the growth of mycorrhizal fungi in a medium containing salts, sugars, B-vitamins and amino acids. Later work by Melin and Rama Das [17]) showed that this "M" factor was produced by a wide range of leguminous and non-leguminous plants. Pea roots, however, also produced an inhibitory factor for the mycorrhizal fungi and the authors suggest that such mechanisms of stimulating and inhibitory excretions are responsible for the specificity of the fungi.

The object of the present investigation was to study the excretion products of oat and pea roots when grown under more natural conditions than those used by many of the above investigators.

EXPERIMENTAL

In this investigation two plant species were used: 1. Field peas (*Pisum sativum*), *var.* Torsdag, 11, and 2. Oats (*Avena sativa*), *var.* Solhavre.

Seed sterilisation

Surface sterilisation of mature and undamaged seed was carried out by a momentary immersion in alcohol, washing with sterile water followed by immersion in 0.2% $HgCl_2$ containing "Tween 80" as wetting agent. The peas were immersed for 15 minutes and the oats for 5 minutes. The seeds were then washed free of $HgCl_2$ with at least six washings of sterile water; they were germinated on potato-glucose agar *) which enables the detection of contaminated seeds. By removing the glumes before treatment almost complete sterilization of the oat seed was obtained. In the initial experiments about 20% of the pea seeds were contaminated in spite of preliminary screening. This was probably due to cracked seed coats and could be considerably reduced by discarding those seeds which were wrinkled or yellow after sterilization.

Cultivation of seedlings

After 4 to 5 days at 25 C the germinated seeds were transferred to sterile 8″×1¼″ tubes containing acid-washed quartz sand and plant-nutrient

*) *Potato-glucose agar.*

A. Potato extract — 500 g chopped potato, boiled in 500 ml water, mashed, filtered and the filtrate made up to 500 ml.

B. 50 g glucose in 500 ml water.

C. 50 g agar in 1500 ml water.

Autoclaved at 110°C for 30 minutes and A, B and C mixed.

solution. The plant-nutrient solution, a modification of Crone's solution (Allen [2]), was prepared as follows:

K_2HPO_4	1.0 g	The salts were mixed and ground. The nutrient solution prepared by dissolving 1.5 g of the mixture in 1000 ml water.
KCl	7.0 g	
$CaSO_4$	2.5 g	
$MgSO_4 . 7H_2O$	2.5 g	
$Ca_3(PO_4)_2$	0.5 g	
$FePO_4$	0.5 g	
KNO_3	2.0 g	

The tubes were placed in racks designed to keep the sand and roots in darkness and placed in a light-room at 15°C ± 5°C with 12 hours illumination and 12 hours darkness. Fluorescent light provided by a mixture of "daylight" tubes and some emitting a redder light was used. These lighting conditions were found to be the optimum, resulting in vigorously growing dark green plants. Fresh additions of nutrient solution were made at weekly intervals when tubes containing contaminated plants were discarded.

Harvesting of plants

For chromatographic analysis one set of plants was removed from the tubes 10 days after planting and the second set after 21 days, *i.e.* 14 and 25 days after the commencement of germination respectively. The technique was to remove the roots from the sand, rinse in distilled water and retain the washings. It should be noted that the roots were only rinsed in the distilled water and not immersed. The sand was then washed several times with distilled water and all the sand and root washings collected. This material was either filtered or centrifuged free of root debris and concentrated under vacuum to give 1 ml of extract for every ten plants.

Desalting of extract for chromatography

The extract contained a high concentration of salts from the nutrient solution and these had to be removed before chromatography for ninhydrin-reacting substances could be performed. The initial technique was that which Boulanger and Biserte [4]) used in removing the salts from blood serum before studying the free amino acids present. The dry material from 5 ml of extract dried under vacuum was suspended in 10 ml of acetone containing 10% 10*N* HCl for one hour, and the supernatant decanted. This extraction was repeated twice and the extracts pooled, dried in the cold under vacuum, and redissolved in butanol-acetic acid-water mixture for spotting on the chromatograms. A desalting method used by Forsman (pers. comm.) in a study of amino acids excreted by excised pea roots was also used in this investigation. In this method, the solution of root exudate containing salts is shaken with an equal volume of phenol and the two phases allowed to separate out. The phenol layer containing the amino compounds is run off, mixed with an equal volume of ether and double volume of water. This is shaken and on separating out, the water solution of the amino acids

is run off leaving the phenol with the ether. This desalted solution was dried under vacuum and redissolved in 0.1*N* HCl to the same concentration as the acetone HCl extract.

In the examination of sugars by unidimensional chromatography the original material was used as the presence of salts did not interfere.

Preparation of standards

The standards used when testing exudates desalted with acetone-HCl were prepared by dissolving pure amino acids in butanol-acetic acid-water, while for material desalted with phenol the standard amino acids were dissolved in 0.1*N* HCl. Acid solvents were used to prepare the solutions of the extracts and standards because of the greater solubility of several amino acids in acid than in neutral solutions.

Chromatography

Descending chromatography at 20°C with Whatman No. 1. paper was used for all the work. For the study of the amino compounds, 80% phenol was the first solvent, with butanol-acetic acid-water (50 : 10 : 10) run in the second direction. The second solvent was allowed to run through the paper for 22 hours to obtain better separation. A comparison of such papers with duplicates in which the butanol-acetic acid-water front had not reached the end of the paper showed that no substances were lost by running this solvent for 22 hours. Identification of the constituents was carried out by comparing the spot intensities of the control root exudate material with those obtained when standard amino acids were added to the material. Approximate estimates of the sugars present were made by running various amounts of the one extract and comparing spot size and intensity with standards containing increasing sugar concentrations. The accuracy of each estimate was increased by including one set of chromatograms containing a constant amount of exudate and increasing sugar concentrations. This is best illustrated as follows:

Composition of spot	Spot number									
	1	2	3	4	5	6	7	8	9	10
ml of extract . . .	.01	.02	.04	.08	.02	.02	.02	—	—	—
μg sugar	—	—	—	—	10	20	40	10	20	40

Duplicates of such series were run for each extract.

Development of chromatograms

For the amino compounds the normal spray of 0.2% ninhydrin in 95%-alcohol was used. For sugars the procedure was as follows, (i) the chromatograms were sprayed with aniline hydrogen phthalate and dried at 105 C for 10 minutes, (ii) These chromatograms then reeeived a spray of 2% orcinol in 2*N* HCl which was allowed to dry at room temperature followed by 90°C for 1 to 2 minutes. The first spray reveals pentoses, aldohexoses and cellobiose

Partridge[9] while the second intensifies these spots and produces bright orange spots with the ketoses or ketone-containing sugars (Forsyth[5]).

All chromatograms were examined for ultra-violet absorption and fluorescent spots with a normal U-V lamp.

RESULTS

(*a*) *Amount of soluble root exudate*

As the root and sand washings contained the residual nutrient solution salts as well as the root excretions it is difficult to give an accurate assessment of the amounts excreted. However, in Table I the total amount of soluble material in 5 ml of extract *i.e.* from 50 plants is given together with the acetone-HCl soluble fraction which is salt free and contains the amino material and probably other organic compounds.

TABLE I

Amounts of material given off by roots during growth in quartz sand

Sample	Total dry substance in 5 ml	Acetone–HCl soluble material in 5 ml	Cell debris from 50 plants
Peas — 10 days	73.7 mg	55.5 mg	18.7 mg
,, 21 ,, ...	89.3 mg	71.6 mg	31.5 mg
Oats — 10 days	37.0 mg	23.7 mg	6.6 mg
,, — 21 ,, ...	50.6 mg	31.8 mg	14.8 mg

(*b*) *Amount of sloughed-off root material*

During extraction of the root exudate from the quartz sand and root washings a suspension of fine cellular material was observed. This material was centrifuged out, dried and weighed to enable an approximate assessment of the relative amounts of root excretions and root debris coming from peas and oats during the early stages of growth. All material which was obviously root fragments broken off in harvesting the roots from the sand was removed before centrifuging and hence is not included in the "cell debris". The results are reported in the final column of Table I.

(*c*) *Amino-acid composition of root exudate*

Although desalting with acetone-HCl was efficient in resulting in clear chromatograms, it caused a partial breakdown of glutamine to glutamic acid and another faint unidentified spot, and also

slight decomposition of asparagine to aspartic acid. The low solubility of some amino acids viz. glutamic, glutamine, cystine, lysine histidine, taurine and arginine in the acetone-HCl may have influenced the relative abundance of these in the extracted material, giving an incorrect impression of the balance of amino acids in the root exudate. The report of Biserte and Scriban[3] that this technique gave ninhydrin-positive artifacts and poor definition of spots was not substantiated, apart from glutamine and asparagine, but it was found that the R_f values were changed in some cases. Because this differential solubility and partial destruction of glutamine and asparagine (with the resultant increase in glutamic and aspartic acids) alters the proportions of amino acids in the extracts, results from chromatograms prepared with the material desalted by the second method are included. However, the need for more phenol-extracted material per chromatogram to obtain spot intensities comparable with the acetone-HCl extract indicated that some losses of amino acids occurred.

The extract obtained by each method revealed some substances not shown by the other, *e.g.* phenylalanine, tyrosine and γ-amino butyric acid in the phenol extract but not in the acetone-HCl which contained the substance giving Spot 13 not apparent when phenol was the extractant. Experiments using pure amino acids indicate that it is unlikely that these substances are artifacts due to the particular desalting technique but probably reflect the different solubilities of the various amino compounds in the solvents.

It is well established that in amino acid chromatography the size and colour intensity of the spot is not necessarily indicative of the concentration, but as the differences in colour intensity of the spots were often large in chromatograms of the exudates, it is considered that the relative concentrations of the amino constituents can be approximately assessed.

The results from the chromatograms are summarized in Table II which also records the probable identity and the relative abundance of each spot in both acetone-HCl and phenol-extracted material.

Eleven of the 27 amino compounds could not be identified with the pure substances available: those tested but not present were: glutathione, taurine, histidine, cystine, proline, arginine and β-alanine. Iso- and nor-leucine were also tested but could not

TABLE II

Identity and relative abundance of the amino-compounds excreted by oat and pea roots

Spot No.	Identity	Pea — 10 days		Pea — 21 days		Oat — 10 days		Oat – 21 days
		Ac–HCl (3.7) *)	Phen. (5)	Ac–HCl (1.5)	Phen. (3)	Ac–HCl (5)	Phen. (20)	Phenol. (20)
1	Asparagine . . .	++	+++	++	+++	—	—	++
2	Aspartic	+++	++	++++	++	++	—	+
3	Serine	++	+++	++++	+++	+	++	+++
4	Glutamic. . . .	++	++	+++	++	—	—	—
5	Glutamine . . .	+++	++++	+++	++++	—	+	+
6	Homoserine . .	++++	++++	++++	++++	—	—	—
7	α-alanine. . . .	++	++	+++	++++	+	+	+
8	Tryptophane . .	+	+/—	+	+/—	—	—	—
9	Methionine or valine	+	+	++	+	+	+/—	+
10	Leucine	+	+	++	+	+	+	++
11	Phenylalanine .	—	+	—	+/—	—	—	+/—
12	? **)	+/—	—	—	—	—	—	—
13	?	+/—	—	+	—	—	—	—
14	?	+/—	+	+/—	+	—	—	—
15	?	+	—	+	+	+/—	+	+
16	?	—	—	+/—	—	—	—	—
17	?	—	+/—	+/—	+/—	—	—	—
18	?	—	—	+	—	—	—	—
19	Lysine	—	++	+	++	+/—	++	+++
20	γ-amino butyric .	—	+/—	—	+	—	—	—
21	?	—	+/—	—	+	—	—	—
22	Tyrosine	—	—	—	+/—	—	—	+
23	?	—	—	—	+/—	—	—	—
24	Threonine . . .	++++	++++	++++	++++	+/—	+	+/—
25	?	—	—	—	+	—	—	—
26	?	—	—	—	—	+	+	++
27	Glycine	—	—	—	—	—	++	+++

*) In parentheses: The number of plants required to excrete sufficient material for chromatographic separation.

**) ? = Unidentified spots.

be distinguished from DL-leucine. Spot 6 has been labelled "homoserine" because its position relative to all the surrounding amino acids is identical with the spot Virtanen, Berg and Kari[29]) termed "homoserine" in their study of the amino-acid composition of germinating pea seeds. When threonine was tested, it corresponded with the lower part of Spot 6 but never with the upper part and hence it was assumed that this large spot contained two substances. The threonine part of the spot has been labelled No. 24. In many chromatograms a constriction almost separating the parts occurred,

supporting the suggestion that the two compounds were present. For reference purposes, the relative positions of all the amino compounds on the chromatograms are shown in Fig. 1.

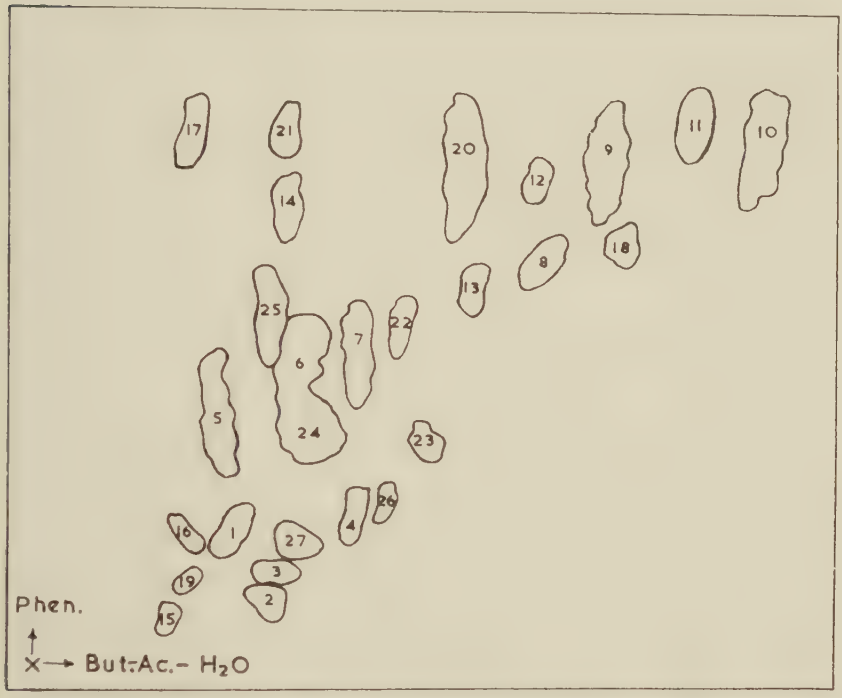

Fig. 1. Composite chromatogram showing the positions of the amino compounds excreted by the roots of peas and oats.

(d) Sugar content of root exudate

The results obtained with unidimensional chromatography and differential developing sprays are summarized in Table III.

TABLE III

Sugars excreted by pea and oat roots during growth (μg/plant)

Source of exudate	Glucose	Fructose	Other sugars
Peas — 10 days	25	25	<5
,, 21 ,, ...	<5	5	<5
Oats — 10 days	20	17	<5
,, 21 ,, ...	<5	<5	<5

(e) Ultra-violet absorbing substances in root exudate

The material from peas after 21 days growth showed two U-V absorbing spots in uni-dimensional chromatograms with butanol-acetic acid-water solvent. The R_f values of these compounds were 0.08 and 0.47. Both compounds were capable of reducing $KMnO_4$ in Na_2CO_3 but not aniline hydrogen phthalate. The reduction of $KMnO_4$ by the substance with R_f 0.47 distinguished it from adenine which has a similar R_f but is non-reducing. The spots did not correspond to guanine or the amino acids tryptophane, tyrosine and methionine which are reported to be U–V absorbing. The compound with R_f 0.47 was insoluble in acetone-HCl as it did not appear after this desalting treatment. Pea root exudate after 10 days contained only one U–V absorbing substance which corre-

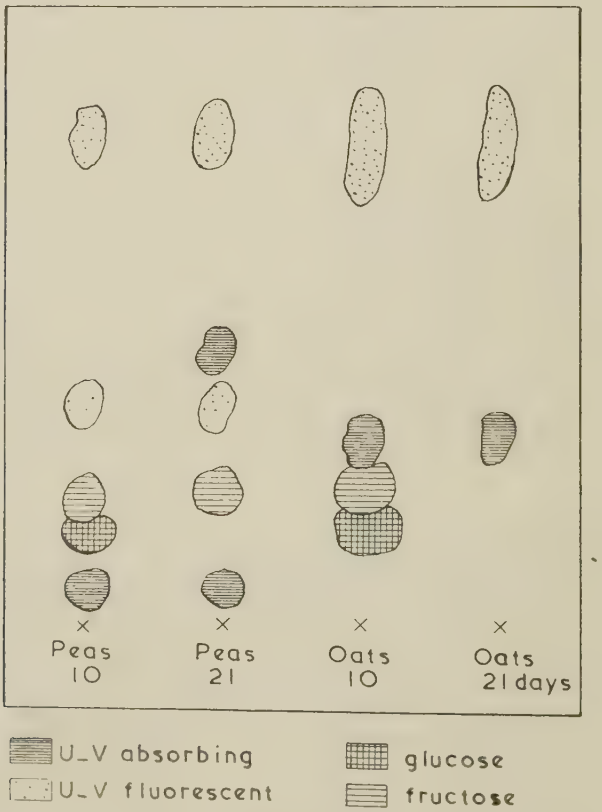

Fig. 2. Sugar and U–V active material in root exudates.

sponded to the low R_f compound present after 21 days. Oats at both ages showed one spot with an R_f 0.30 in butanol-acetic acid-water and soluble in phenol. This substance was present in greater amounts after 10 days than after 21 days.

(f) Ultra-violet fluorescent constituents of root exudate

All the plants excreted material which produced fluorescent spots with U-V light but no attempt was made to identify them. The positions of these spots relative to the glucose, fructose and U-V absorbing spots in one-dimensional chromatograms with butanol-acetic acid-water solvent are shown in Fig. 2.

DISCUSSION

The results indicate that at least for the first three weeks of growth of both peas and oats actual excretions form the bulk of the material coming from the roots, and hence must play an important role in the stimulation of the micro-organisms on and around the roots. Some of the soluble material is probably not excreted in the true sense, resulting from the autolysis of sloughed-off cells, but as it is impossible to separate these two sources all the soluble material from the root is regarded as exudate. The assumption that the bulk of this exudate is true excretion and not the products of autolysis is supported by the observation that, although the amount of cell debris for both oats and peas is doubled between 10 and 21 days, the amount of soluble material is only increased by half. However, it is interesting to note that while at 10 days the dry weight of material excreted is three to four-fold the dry weight of root debris, at three weeks the amount of root exudate is little more than double that of the sloughed-off cells. This indicates that, as the plants develop, true root excretions are probably not as important in influencing the rhizosphere population as is the cast-off cell material. These quantitative studies are only approximate as a substantial amount of cell debris undoubtedly remained firmly attached to the quartz sand. With this change in the nature of material coming from the roots as they develop, it is conceivable that the rhizosphere population may change from one predominantly requiring amino acids to one requiring the more complex substances derived from the sloughed-

off material. The nutritional classification of Lochhead and Chase[12]) by which they found a selective stimulation of amino acid requiring bacteria in the rhizosphere has been disputed by Rovira[25]) and Wallace and King[31]). It appears possible that the age of the plant at sampling could be one explanation for these different findings. However, although the dry weight of the acetone-HCl soluble fractions increased from 55.5 mg to 71.6 mg per 50 plants between 10 and 21 days, the amount of material required per chromatogram for good spot separation and intensity was equivalent to 3.7 plants for 10-day plants and 1.5 for 21-days plants. Thus while total root excretions increased by only 30%, the amount of amino material increased by over 100%, indicating that the older plants excrete a material richer in amino compounds. These results are confirmed by the spot intensities obtained when the material was desalted with phenol.

The chromatograms indicate qualitative as well as quantitative changes in the root exudate with age. In peas, not only have new amino compounds appeared (Spots 15, 16, 22, 23 and 25) but the relative concentrations of the others have changed. As the yield of root exudate at 21 days included the substances which have accumulated over the whole period *i.e.* those recorded at 10 days, any changes in the nature of the exudate during the second 10 days of growth must be sufficiently great to overshadow the earlier composition of the exudate. Thus, while homoserine, threonine and glutamine were the most abundant at both ages, the exudate after 21 days was relatively richer in asparagine and alanine. Also, there were changes in the concentrations of other constituents, viz. γ-amino butyric acid and Spot 21, with growth. Similarly, as oats developed, changes in the exudate occurred — after 10 days the exudate was richest in lysine, serine and glycine while by 21 days the predominant amino acids were lysine, glycine, serine, asparagine and leucine.

The appearance of new substances with age does not necessarily mean that they have been excreted by the plants only after 10 days. It is possible that trace amounts of these substances have been excreted continuously since germination and only after the longer period did the concentrations become sufficiently great to detect. However, the findings of Virtanen, Berg and Kari[29]) with germinating peas, and Biserte and Scriban[3]) with

barley, that there is a qualitative change in the free amino acid content of the seed and plant during germination and early growth make it conceivable that the amino-acid composition of the exudate may change with age.

When Wallace and Lochhead[32]) showed that the growth requirements of 80% of the amino-acid dependant rhizosphere bacteria could be satisfied by supplying methionine as the sole amino acid, the suggested that methionine may be one of the major amino acids excreted into the rhizosphere. This may be so in other plants or even in these species at a later stage, but the evidence obtained in the present investigations shows methionine (if it is present, as the spot could be due to valine which is inseparable in the solvents used) to be only a minor excretion product. The other sulphur-containing amino acids, cystine and taurine, which in some cases can replace methionine in the nutritional classification of Lochhead and Chase[12]) were not found in the exudates.

The pea root exudate differed quantitatively and qualitatively from that of oats — after 21 days the peas had excreted 22 amino compounds and oats 14 and the extract from 20 oat plants was necessary to give spots with approximately the same intensity as the extract from 1.2 pea plants. Not only did the total amount of material and numbers of amino compounds differ between the two species, but also the proportions of the constituents. In peas at 21 days the most abundant amino acids were homoserine, threonine, glutamine, asparagine and alanine while in oats at the same age glycine serine and lysine appear the most abundant followed by asparagine and leucine.

When the sugar content of the exudates was studied, the oats and peas were each found to excrete glucose and fructose during the first ten days of growth. After 21 days, however, the sugars had disappeared from the root exudate indicating a re-absorption or a synthesis of oligosaccharides from the original excreted sugars Although the quantities of sugars excreted per plant were low (maximum 25 microgram) this energy source undoubtedly has a stimulating effect on the soil population during the early stages of growth.

The presence of ultra-violet absorbing compounds has been recorded in this study but they were not identified. As several

workers, Lundegårdh and Stenlid [14]), Stenlid [26]) and Fries and Forsman [9]) have reported the excretion of nucleic acid derivatives from excised roots, the presence of U-V absorbing substances in exudate from roots of actively growing plants is not surprising.

Several spots were found which fluoresced in U-V light but their identity was not established. There are a wide range of substances which fluoresce in this way including glycosides and tannins found in potato tubers.

Although only the amino compounds and sugar constituents of the root exudate were considered in detail, it is still possible to explain the development of a characteristic flora around the roots of plants of different ages and different species. Besides the sugars and amino compounds, there are the growth factors and other substances in the exudate and the products of decomposition of sloughed-off cells which will influence the nature of the rhizosphere population even further. Other results obtained in nutritional studies on soil and rhizosphere isolates by the author indicating that constituents other than amino acids in the pea and oat root exudate are responsible for maximum growth of the isolates serve to show the complexity of the material excreted by plant roots *).

SUMMARY

The excretory products of roots of peas and oats grown aseptically in quartz sand were studied by paper chromatography. Peas were found to excrete considerable amounts of amino material during 21 days growth with 22 different amino compounds, while oats excreted less consisting of 14 amino compounds. The proportions of the various amino acids in the exudates differed between peas and oats. Fructose and glucose were excreted by both plants only during the first ten days of growth. U–V absorbing and fluorescent compounds were also excreted. The approximate amounts of sloughed-off cell material from both peas and oats after 10 and 21 days were also estimated.

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*) *Addendum:* Since the completion of this investigation, Parkinson (Nature **176**, 35) has also reported the excretion of amino acids from oat seedlings grown aseptically.

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PLANT ROOT EXCRETIONS IN RELATION TO THE RHIZOSPHERE EFFECT

II. A STUDY OF THE PROPERTIES OF ROOT EXUDATE AND ITS EFFECT ON THE GROWTH OF MICRO-ORGANISMS ISOLATED FROM THE RHIZOSPHERE AND CONTROL SOIL

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INTRODUCTION

Although there are many reports on the excretion of organic substances by roots, little has been done on the effects of these excretions on micro-organisms isolated from soil and rhizosphere. West [12]) demonstrated that the root excretions of flax could supply the thiamin and biotin requirements of *Micrococcus aureus* and *Rhizobium trifolii* respectively, but did not study the effects of the exudates on soil organisms as a group. Katznelson, Rouatt and Payne [5]) found that the growth requirements of the amino acid-dependent group of rhizosphere bacteria could be satisfied with supplements obtained from the leachings of sand in which plant roots had been grown. In a study of the growth of bacteria in complete nutrient solution with and without plants, Meshkov and Khodokova [7]), found that the presence of the roots of peas and oats favoured non-sporing organisms more than spore formers. They reported that the excretions of pea roots were more favourable to *Azotobacter* than were those from maize roots.

An investigation by the author [11]) of the quantitative and qualitative nature of the excretions from the roots of oats and peas showed that a wide range of substances was excreted. It was

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considered that a study of the growth responses to these exudates by bacteria isolated from the rhizosphere and normal soils may indicate the mechanism whereby the roots selectively stimulate the micro-organisms in the rhizosphere. The results of the investigation are presented in this paper.

EXPERIMENTAL

Source of isolates

The 97 cultures used in this study were isolated from colonies selected at random from the highest dilution plates prepared from soil and rhizosphere samples using the medium of Bunt and Rovira[1]). After purification the isolates were maintained on this medium with 0.1% glucose. Isolates from the rhizosphere were obtained from the roots of five-week pea plants The $R : S$ ratio of seven indicates that the isolates were representative of the root surface population. The control soil samples were obtained from fallow pots which had received the same treatment as those in which the peas were grown.

Preparation of root exudate

The method of Rovira[11]) was used except that the final solution obtained was concentrated to 1 ml per plant.

Media for testing the activity of root exudate

A wide range of media of varying complexity was used, the composition of the medium depending on the aims of the particular experiment. When testing the root exudate for its ability to promote the growth of soil and rhizosphere isolates, relatively simple media containing basal salts and either glucose or soil extract, or both, were used. The more complex media with amino acids, yeast extract and vitamins were used when seeking substances with similar activity to root exudate. Comparisons were made of the growth of organisms in these media with and without additions of root exudate. The concentration of exudate used varied between 0.14 and 0.028% depending on the experiment.

The basal medium (No. 1) used throughout the experiment had the following composition:

K_2HPO_4	0.4 g.
$(NH_4)_2HPO_4$	0.5 ,,
$MgSO_4.7H_2O$	0.05 ,,
NaCl	0.1 ,,
$CaCl_2$	0.1 ,,
$FeCl_3$	0.01 ,,
Water.	1 litre.

The other media used in this investigation were:

No. 2:	Basal medium	+ 0.1% glucose.
No. 3:	,, ,,	+ 25% (v/v) soil extract.
No. 4:	,, ,,	+ 0.1% glucose + 25% soil extract.
No. 5:	,, ,,	+ 0.1% peptone + 0.1% yeast extract + 25% soil extract.
No. 6:	Medium 2	+ 0.05% casamino acids *)
No. 7:	,,	+ 0.1% casamino acids
No. 8:	,,	+ 0.05% Difco yeast extract
No. 9:	,,	+ 0.1% Difco yeast extract
No. 10:	,,	+ 0.05% casamino acids + 0.05% yeast extract
No. 11:	,,	+ 0.1% casamino acids + 0.1% yeast extract
No. 12:	,,	+ 0.1% asparagine
No. 13:	,,	+ vitamin mixture **)
No. 14:	,,	+ 0.05% casamino acids + 0.05% yeast extract + 0.1% asparagine + vitamins.

Inoculation

The inoculum for each test consisted of one drop of dilute suspension prepared from the growth on an agar slope after five days at 24°C.

Assessment of growth

The growth of the organisms in the various media was assessed visually after 5 days at 25°C. The visual ratings were from 0 (no growth) to 5 (less than 25% transmission).

Testing the stability of pea root exudate

Preliminary experiments were carried out testing the heat stability of the exudate by measuring the activity after the following treatments:

(i) Steaming for 1 hour on each of three successive days.
(ii) Autoclaving at 105°C for 60 minutes.
(iii) ,, ,, 110°C ,, 30 ,,
(iv) ,, ,, 120°C ,, 20 ,,
(v) ,, ,, 130°C ,, 20 ,,

The stability of root exudate to dialysis, various extractants (charcoal, alumina, chloroform, and benzene), and to acid (6*N* HCl) and alkaline (6*N* NaOH) hydrolysis at 120°C for one hour, was also tested. In this part of the work three rhizosphere isolates, selected for their marked responses to root exudate, were used to test the growth-promoting properties of the exudate in basal salt-glucose medium after treatment.

*) *Casamino acids* — Difco "Vitamin-free casamino acids" supplemented with tryptophane, cystine, serine and aspartic acid.

**) *Vitamin mixture* — Thiamin (10 μg), riboflavin (20 μg), pyridoxine (20 μg), nicotinic acid (10 μg), biotin (0.01 μg), inositol (1 mg) per 100 ml.

Incorporation of root exudate into counting medium

The effect of incorporating root exudate (0.14% w/v) in the medium for estimating the numbers of organisms in control and rhizosphere soils was studied by adding it to the agar medium before sterilization, and comparing counts obtained in this medium with those in the control without root exudate.

RESULTS

(*a*) *The growth promoting action of various concentrations of root exudate*

The optimum concentration of root exudate necessary for maximum response by a maximum number of organisms was studied in two media, basal salts — glucose and basal salt-soil extract, with three concentrations of pea root exudate, viz. 0.14, 0.056 and 0.028%. Table I shows the number of cultures, from 26 tested, which responded to the supplements.

TABLE I

No. of rhizosphere isolates (of 26 tested) which responded to root exudate		
Concentration of root exudate (*w/v*)	Test medium	
	Basal + glucose	Basal + soil extract
0.14 %	24	25
0.056%	20	20
0.028%	5	6

From these results it could be seen that in order to obtain a maximum number of isolates responding to root exudate supplements, the highest concentration should be used. At this concentration the responses by the individual cultures were, in most cases, greater than at lower concentrations.

(*b*) *Responses of soil and rhizosphere isolates to root exudate*

In Table II are reported the turbidity readings of nineteen soil and nineteen rhizosphere isolates in several media with and without root exudate.

The turbidity results are reported in detail to enable a comparison of the responses of individual organisms to the various supplements including root exudate. The different responses of the isolates to

TABLE II

Culture No.	Responses of soil and rhizosphere isolates to root exudate									
	Medium 1		Medium 2		Medium 3		Medium 4		Medium 5	
	—	+	—	+	—	+	—	+	—	+
Soil isolates										
99	0	1	0.5	1	0.5	1				
4	0.5	1	3	5	1	2				
80	0	1	0	1	0	1				
16	0	0	0	0.5	0	1				
67	0	0.5	0	1	0	2				
97	0	1	0	0.5	1	2				
57	0	1	3	5	1	2				
95	0	1	0	1	0.5	1				
30	0	1	2	4	0.5	2				
38	0	1	0.5	0.5	0.5	1				
98	0	1	0	1	1	2				
32 *)	0.5	2	4	5	2	3				
73	0.5	1	3	4	2	3				
76	0	1	0.5	1	0.5	1				
40	0	1	0	1	1	2				
31	0	0.5	1	0.5	0.5	0.5				
52 *)	1	0.5	3	2	3	3				
65	0.5	1	3	5	1	2				
39	0	1	0	1	0.5	0.5				
Rhizosphere isolates										
51	0	1	1	2	1	3	1	2	3	5
62	0	2	4	5	1	3	5	5	4	5
18	0	2	3	5	1	3	3	5	3	5
68	1	2	1	2	1	2	2	2	3	5
99	0	2	4	5	1	2	3	5	4	5
67	1	2	4	5	2	3	3	5	3	4
83	0	3	3	3	2	3	2	5	3	4
32	1	2	3	5	3	4	5	4	4	5
85	0.5	3	4	5	1	3	4	4	4	5
12	0.5	3	4	5	1	3	3	5	3	5
64	0	3	3	4	0	3	3	3	4	5
97	0	2	4	5	0.5	2	1	2	3	4
58	0	1	0	1	1	2	3	5	4	5
66	0.5	2	5	5	1	2	1	3	3	4
74	0.5	1	0.5	2	1	2	4	3	4	5
98	0.5	1	4	5	1	2	0	2	4	5
86	0.5	1	0.5	3	0.5	3	1	2	4	5
19	0	1	0	3	0	1	4	5	3	5
17	1	3	4	4	1	3	2	4	4	5

*) Cultures 32 and 52 were unidentified actinomycetes.
— = without root exudate (control).
+ = with root exudate.

the media illustrate the wide variability in their nutritional requirements and the difficulty of applying a nutritional classification to soil microorganisms.

The main point arising from the table is that both groups of organisms were stimulated by root exudate. The fact that two of the soil isolates failed to respond to root exudate while all from the rhizosphere were stimulated is probably not significant. In view of the fact that the rhizosphere population of young plants is primarily due to the root excretions and that a selective stimulation occurs in the rhizosphere, it is at first surprising that a general stimulation of both groups of isolates should occur. However, consideration of the degree of response in Medium 3 reveals that there was a mean response of 0.8 units to the root exudate supplement by the soil isolates and 1.6 units by the rhizosphere cultures. The soil extract medium was selected for this comparison as it is considered the closest to the soil environment and the response in this medium may be indicative of how the organisms would respond in the rhizosphere.

One point made clear in the table is that the isolates from the rhizosphere generally gave more prolific growth than those from control soil in similar media; *e.g.* in Medium 2+, six of the 19 soil isolates gave readings of three or more, whereas 16 of the 19 rhizosphere organisms gave such readings. Earlier, when obtaining pure cultures, it had been observed that growth on agar media was slower and less prolific for the soil organisms than for those from the rhizosphere. This indicates that the roots selectively stimulate organisms capable of responding readily to the nutrients liberated and it is probable that the six organisms from the control soil which grew abundantly would be the soil organisms to proliferate in the rhizosphere.

(*c*) *Comparative responses of pea rhizosphere and soil isolates to exudates from peas and oats*

It was considered that a study of the responses by soil and pea rhizosphere organisms to exudates from the roots of peas and oats may show a greater stimulation of the rhizosphere isolates by the pea root exudate. However, the results (Table III) indicate that both exudates stimulated the growth of both groups of organisms in basal salt-glucose-soil extract medium.

TABLE III

Responses of control soil and pea rhizosphere isolates to pea and oat root exudates (Growth rating 0 to 5)

Soil isolates			Rhizosphere isolates		
Medium 4 (control)	+ Oat root exudate	+ Pea root exudate	Medium 4 (control)	+ Oat root exudate	+ Pea root exudate
1	4	4	1	3	2
2	4	3	1	4	4
1	2	3	0.5	1	3
0.5	3	3	3	3	5
1	2	2	0.5	4	3
0	1	1	1	2	4
0.5	1	1	1	5	5
1	3	2	4	5	5
1	3	3	1	2	2
1	2	3	3	5	4
1	3	2	0	1	1
0.5	2	1	2	5	3
0	1	1	1	3	3
0	1	2	0	3	1
0	1	0.5	3	5	5
0	1	1	2	5	5
1 *)	3	3	1	3	1
0.5	2	1	3	4	5
1 *)	3	3	2	2	4
0.5	1	2	1	3	3
0.5	3	1	3	5	4
1	3	2	2	2	3
1	3	2	3	4	5
1	2	3	3	5	5
0	1	1	2	5	4
4 *)	4	4	3	5	4

*) Unidentified actinomycetes.

As in Table I, these results show more abundant growth by the rhizosphere organisms and a general response by both groups of organisms to root exudates. The stimulation by both exudates was generally of a similar order with some organisms responding slightly more to one than the other. This rather unexpected result following the finding that pea roots excreted a higher concentration and greater number of amino acids than did oats (R o v i r a [11]) indicates that the growth promoting action of the exudates is largely due to constituents other than amino acids.

(*d*) *Activity of pea root exudate in media of varying complexity*

The cultures used in this part of the investigation originated

from the pea rhizosphere and were selected because their different responses to supplements and root exudate indicated varying nutritional requirements.

TABLE IV

The growth of six rhizosphere cultures in media of varying nutritional complexity with and without root exudate
(Growth rating 0 to 5)

Supplement to Medium 2	Culture No. 18		32		57		58		64		74	
	—*)	+	—	+	—	+	—	+	—	+	—	+
Nil	2	3	2	4	2	4	0	1	1	2	1	2
Amino acids (0.05%)	2	4	2	5	2	4	1	2	2	2	1	4
Amino acids (0.1%)	2	4	1	3	1	4	2	3	2	3	1	4
Yeast extract (0.05%)	3	5	4	4	4	4	3	4	3	3	3	4
Yeast extract (0.1%)	3	5	4	4	4	5	4	4	3	5	4	5
Amino acid (0.05%) + Yeast ext. (0.05%)	3	4	4	4	3	4	3	3	2	3	2	4
,, ,, (0.1%) + ,, ,, (0.1%)	3	5	4	4	4	4	3	5	3	4	2	4
Asparagine (0.1%)	1	4	1	4	2	4	0	1	1	1	1	4
Vitamin mixture	2	4	2	4	2	4	0	1	2	2	2	3
Complete mixture	3	5	4	4	3	4	3	5	3	5	3	4

*) — = without root exudate, + = with root exudate.

In the results, cultures 32 and 57 responded equally to yeast extract and root exudate suggesting common constituents. The failure of vitamin-free amino acids to stimulate the growth of these organisms demonstrates that the growth promoting properties of yeast extract and root exudate are due to constituents other than amino acids. The further stimulation of growth of cultures 18 and 74 by root exudate when added to media containing yeast extract, reveals that the root exudate supplies factor(s) not supplied by yeast extract. If this stimulation had occurred at the lower concentration of yeast extract only, it could have been due to an increase in the supply of constituents common to both root exudate and yeast extract. However, there was still a significant increase in growth with the root exudate at the higher concentration of yeast extract. The two remaining organisms responded to the amino acid supplements due to their inability to synthesize their amino acid requirements and were further stimulated by yeast extract and root exudate.

Asparagine, which is excreted by pea roots, had no stimulating effect when added to the basal medium, indicating that in these pure

culture studies asparagine was not one of the active constituents of the exudates. In the cases of organisms 18 and 32 asparagine partly inhibited growth, a result which is difficult to account for.

The vitamin mixture containing thiamin, riboflavin, pyridoxine, nicotinic acid, pantothenic acid, inositol, and biotin, failed to improve growth over the controls in four cases and only slightly in the remaining two. In this respect it is interesting to compare the vitamin composition of the yeast extract and the synthetic mixture as shown in Table V.

TABLE V

Micrograms of vitamin per ml of culture media		
Vitamin	Synthetic mixture	"Difco" yeast extract *) (0.1% level)
Thiamin	0.1	0.0032
Riboflavin	0.2	0.019
Pyridoxin	0.2	0.02
Nicotinic acid	0.1	0.279
Biotin	0.0001	0.0014
Inositol	10	No figure available

*) Analysis provided by Difco Laboratories.

With the exception of biotin and nicotinic acid, the vitamin supply of the synthetic mixture is considerably greater than in the yeast extract which makes it unlikely that the failure of the former to stimulate growth is due to the vitamin supply. A comparison of the concentration of growth factors in the synthetic mixture with the vitamin requirements of a wide range of organisms listed in Porter[10]) shows that in most cases the supply would be adequate. It is more probable that the yeast extract supplies growth factors not listed in the analysis data, some of these also being present in the root exudate. The lack of response by the cultures to amino acid supplements alone and in the presence of yeast extract is further evidence that the growth factors, rather than the amino acids, are responsible for its growth promoting properties.

(*e*) *The stability of pea root exudate*

(i) Effect of heat. The growth ratings of nine rhizosphere isolates in basal medium with pea root exudate after various heat treatments presented in Table VI show that the growth promoting effect of the exudate ware increased with heating.

TABLE VI

The growth of rhizosphere organisms after heat treatment of root exudate (Growth ratings 0 to 3)

Culture	Heat treatment				
	100°C, 3 hours	105°C, 1 hour	110°C, 30 min	120°C, 20 min	130°C, 20 min
17	2	2	2.5	2	2.5
18	2	2	2	2	2.5
19	2	2	2	2	3
32	2	2	2	2.5	3
57	2	2	2	2	3
62	2	—	2	2.5	3
74	2	2	2	2	3
83	2	2	3	3	2
85	1.5	2	2.5	3	3

The growth of the cultures in this experiment was never more than moderate (rating 3) as only basal salt medium was used with the root exudate. This was done in order to have a medium in which the only possible heat-labile constituent was the exudate. This tendency for the activity of the exudate to increase with severity of treatment was probably due to a partial breakdown of some of the constituents into more readily available compounds.

(ii) Other properties of the pea root exudate. Among the properties tested, was the stability of the exudate to acid and alkaline hydrolysis, the results of which are reported in Table VII.

TABLE VII

Activity of pea root exudate after heating with 6*N* HCl and 6*N* NaOH (Growth rating 0 to 5)

Culture	Control *)	Acid treatment	Alkali treatment
32	4	5	3
57	3	4	3
74	3	0	1

*) Untreated root exudate.

The interesting feature of these results is the varied responses by the three organisms to the treated exudate due to their different nutritional requirements. Organism 74 was obviously dependent on some factor which was destroyed by both treatments while the other organisms, which had shown similar properties in earlier studies,

gave a greater response to the exudate after acid treatment than did the control. Alkaline hydrolysis slightly reduced the activity of the exudate for organism 32.

The activity of root exudate following dialysis, extraction at pH 5.5 with solvents such as chloroform and benzene, and extraction with the adsorbants, charcoal and alumina, was tested and the results are given in Table VIII. These results are further evidence of the complexity of the exudate as the three organisms responded differently to the fractions after treatment.

TABLE VIII

Effect of extraction and adsorption on the activity of root exudate (Growth rating 0 to 5)

Culture	Treatment					
	Control *)	$CHCl_3$	Benzene	Charcoal	Alumina	Dialysis
32	5	4	5	1	3	2
57	5	3	4	1	2	1
74	4	4	4	2	2	1

*) Untreated root exudate.

(*f*) *The effect of root exudate in counting media*

The demonstration of the stimulating activity of root exudate on the growth of soil and rhizosphere isolates in liquid media suggested that the incorporation of the exudate in agar media may increase plate counts, particularly in the rhizosphere samples where the population is largely due to the excretion of such material from the roots. The mean number of colonies per plate at the highest suitable dilution with and without root exudate are shown in Table IX.

TABLE IX

The effect of root exudate on the plate count of rhizosphere and control soils

Expt.	Sample	Medium	
		Control	With exudate
1	Control soil	67 *)	77
	Rhizosphere	82	99
2	Control soil	57 **)	60
	Rhizosphere	124	135

*) Means from four replicate plates in Expt. 1.
**) ,, ,, eight ,, ,, ,, ,, 2.
S.E. = ± 9.5%.

Although there was a tendency for the exudate to increase counts, it was not significant, which substantiates earlier findings obtained with a simpler plating medium (unpublished). This result is not in contradiction with those reported in Tables I and IV, as all the organisms were capable of sufficient growth in the absence of root exudate to produce visible colonies in the agar medium.

DISCUSSION

The observation that rhizosphere organisms grow more abundantly in pure culture than do soil isolates indicates that they may be present on the root surface largely as the result of their ability to multiply more rapidly than the majority of the normal soil microflora. This is supported by the finding that the mean degree of response to root exudate by the rhizosphere organisms is considerably greater than that of the organisms from the control soil. This greater response by rhizosphere organisms suggests that they probably belong to the "Zymogenous" group of W i n o g r a d s k y and are normally present in soil in low numbers but multiply rapidly with additions of readily decomposable organic matter.

The general stimulation of soil and rhizosphere organisms by root exudate suggests that given suitable conditions, most soil organisms would be capable of utilizing the exudate and establishing themselves on the root surface. However, under normal field conditions with strong competition for the excretory products, those organisms capable of the most rapid responses will predominate on the root surface. Slight differences in the responses of organisms to the exudate in pure culture will probably be magnified considerably on the root surface where the exudates will be utilized immediately they are excreted, allowing no opportunity for accumulation. Another important factor influencing the predominance of particular organisms in the rhizosphere will be the ability of the stimulated organisms to antagonise other groups, but, even omitting this effect, the different responses by soil isolates to root exudate seems sufficient to explain the selective stimulation in the rhizosphere.

The stimulation of pea rhizosphere organisms by both pea and oat root exudates does not necessarily mean that these plants will have the same root surface flora. The results have shown that

the responses by the isolates to the two exudates differ; in some cases the pea root exudate having the stronger effect and in other cases the oat root exudate. As suggested above, these effects will be even more marked in the rhizosphere, leading to different populations on the two plants.

An examination of the growth of rhizosphere isolates in the various media listed in Table II shows that all grew in the peptone-yeast extract-soil extract medium while several failed to grow on the simpler mineral salts-soil extract medium. Thus it is obvious that the use of the former medium for estimation and isolation of soil organism should yield the greater number.

In the study of the concentration of root exudate required in media to stimulate the organisms, it was found that a rather high concentration (0.058% to 0.14%) was required, which suggested that it was acting as an energy or amino acid source rather than supplying growth factors which are required at a lower level. However, later findings showing greater responses by most isolates to root exudate in the media containing glucose and vitamin-free amino acids than in yeast extract media indicates that the growth factor content was mainly responsible for the activity of the exudate. The lack of response to a mixture of known vitamins indicates that the factors which root exudate and yeast extract have in common belong to the unidentified fraction of the latter. It has been shown in many cases that the stimulating action of yeast extract is due to unidentified factors (Peterson and Peterson[9]), Omata[8]) and Hendlin[3])). As the purines and pyrimidines, which have been shown to act as growth factors (Porter[10]), Hendlin[3])), are excreted by excised roots (Lundegårdh and Stenlid[6]) Fries and Forsman[2])), they were probably partly responsible for the activity of the exudates used in this investigation.

The study of the stability and properties of pea root exudate serves to indicate that it comprises several active constituents with different properties. This prevents comparison of the properties with those of other unidentified constituents described by Kline and Barker[4]) and Omata[8]). Fractionation of the exudate and a study of the properties of the various fractions seems desirable in an attempt to obtain more specific information on the nature of the active constituents.

SUMMARY

The inclusion of exudates from the roots of peas and oats in liquid media has been found to increase the growth of micro-organisms isolated from both control soil and the rhizosphere of three-week old pea plants. In most cases the stimulation was greater for organisms from the rhizosphere than for those from outside this zone. For some organisms, yeast extract exhibited similar growth promoting properties as did pea root exudate, but for others the exudate stimulated growth in the presence of yeast extract. The root exudate could not be completely replaced by glucose, soil extract, vitamin-free casamino acids, or a synthetic mixture of known growth factors.

The effects of temperature treatment, acid and alkaline hydrolysis, extraction with chloroform, benzene, charcoal and alumina, and dialysis on the growth promoting activity of pea root exudate have also been studied.

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